

Archives of Oral Biology

Volume 5 1961

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Publishing Offices: Headington Hill Hall, Oxford (Oxford 64881)

Annual subscription (including postage):

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Headington Hill Hall,
Oxford

122 East 55th Street,
New York 22, N.Y.

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AN EXPERIMENTAL INVESTIGATION OF TISSUE CHANGES ASSOCIATED WITH DERMAL AND MUCOSAL CONTACT OF EPOXY RESINS AND THEIR CURING AGENTS

S. ÖSTLUND, G. BJÖRLIN and G. GUSTAFSON

Departments of Prosthetic Surgery and Histopathology,
Royal Dental School, Malmö, Sweden

Abstract—In an investigation of the skin and mucosa of rabbits and dogs to study the effect of contact with non-polymerized and polymerized epoxy resins and with their curing agents (diethylenetriamine), severe tissue changes and necrosis were observed. The lesions were not due to the resin but to the curing agent. The non-polymerized epoxy resin produced a thickening of the keratinized layer of the epithelium.

AMONG the new materials introduced for use in dental prosthetics, epoxy resins are of particular interest. They were early used as a dental material, but to-day constitute an industrial product of great importance. Owing to the lack of a non-toxic and rapid catalyst, the material cannot yet be used as a tooth filling material. Epoxy resin has, however, been tried where a short curing time is not essential as, for instance, in making dentures and dies.

The dental literature on the use of epoxy resins is scanty. BOWEN (1956) carried out experiments *in vitro* with epoxy resins as filling materials, and concluded that these resins might well be used clinically. KYDD and WYKHUIS (1958) recommended epoxy resins as substitutes for acrylic resin in making dentures. ÖSTLUND and ÅKESSON (1960), however, found epoxy resin to have a lower transverse strength than methyl methacrylate. KYDD and WYKHUIS also considered the possibility of these resins causing allergic reactions. In a series of experiments in which epoxy resin was applied to the arms of fifty students, reactions of varying types were observed in sixteen. Tissue damage has also been reported among people working with epoxy resins in industry. GRAND-JEAN (1955) found as many as 43 per cent of such workers to have dermatosis. This was mild in 21 per cent and severe in 22 per cent. These observations were verified by BOURNE (1957) who suggested certain prophylactic and therapeutic measures.

The purpose of the present experimental investigation was to determine whether epoxy resins or their curing agents produce histologically demonstrable changes or mucosal reaction.

MATERIALS AND METHOD

(a) *Skin test*

These experiments were carried out on two rabbits.

The substances to be tested were placed in special "cups" made in the following way. In the centre of round 0.5 mm thick tin plates, about 25 mm in diameter, a semi-spherical impression was made with an inner diameter of 8 mm and a depth of 3.5 mm (Fig. 1). In the margin of the plate a number of holes were made to receive sutures to hold the plate against the skin. Twelve cups were made and six sutured on each animal. Four of the cups were filled with a freshly prepared mixture of epoxy resin (Epicote 815, Shell Chemical Corp., New York, N.Y.) and a curing agent, diethylene triamine (DETA). The cups were placed horizontally and the mass was allowed to polymerize for 24 hr. Two cups were filled with un-polymerized epoxy resin, one in a vaseline base and the other in a mucilan base (carboxy methylcellulose). Finally, two cups were filled with the curing agent (DETA), one in a vaseline base and the other in a mucilan base. To check whether any of the ointment bases might, by themselves, be capable of causing any skin reaction, the remaining cups were filled with carboxy methylcellulose, vaseline and paraffin. The backs of the animals were shaved, and the cups sutured to the skin, three on either side of the mid-line. The cups were placed about 5 cm apart. After 8 days, the animals were killed and the part of the skin in direct contact with the contents of the cups were excised and studied histologically.

(b) *Mucosal tests*

These experiments were carried out on two dogs.

Alginate impressions of the upper jaws of the dogs were taken in special trays and a base plate made for each dog. One of the plates was made of methyl methacrylate and the other epoxy resin (PermaRock, Permament Products, New York 19, N.Y.). Before curing the baseplates, strips of 1 mm tin foil, 10 mm long and 5 mm wide, were placed in pairs on either side of the mid-line in the spaces between the crescent-shaped palatal rugae (Fig. 2). In this way, symmetrical recesses were obtained in the base plate. The test material (Table 1) was placed in these recesses. The epoxy resin Epicote 815 was used with DETA as curing agent and paraffin as a base for the mixture. Paraffin alone was placed in those cavities marked "control". The ventral and dorsal parts of the plates served as control areas for polymerized methyl

TABLE 1. BASE PLATES CARRYING DIFFERENT KINDS OF TEST MATERIAL

Dog 1. Base plate in acrylic resin		Dog 2. Base plate in epoxy resin	
Control	Epoxy resin	Control	Control
Curing agent (amine)	Control	Epoxy resin	Epoxy resin
Control	Epoxy resin	Control	Curing agent (amine)
Curing agent (amine)	Control	Curing agent (amine)	Control
Control	Epoxy resin	Control	Curing agent (amine)

methacrylate and epoxy resin respectively. The plates with the various test substances in the recesses were placed into the mouths of the dogs and held in position by stainless steel wire ligatured to the teeth. The acrylic plate was removed after 4 days; the epoxy resin plate after 6 days. When the plates had been removed, the animals were killed and the entire oral mucosa excised. Those pieces of mucosa covered by the recesses in the plates were cut out, fixed in 10% formalin and paraffin sections prepared.

RESULTS

(a) *Skin tests*

Only the areas which had been in contact with the amine curing agent showed any reaction. This reaction was extremely severe with necrosis of the entire surface layer. The necrotic tissue was bordered by a layer of inflammatory cells, mainly polymorphonuclear leucocytes, parallel to the surface (Fig. 3). New epithelium in which a high mitotic rate could be observed was growing in from the outer edge of the damaged area (Fig. 4). Immediately beyond the areas of necrosis the epidermis showed changes and the keratinized layer was absent.

(b) *Mucosal tests*

The mucosal changes in the dog varied widely and ranged from necrosis to hyperkeratosis. Where the amine curing agent had been in contact there was considerable necrosis of the surface layer of epithelium. Sometimes the epithelium contained vesicular formations (Fig. 5, a and b) containing inflammatory cells, mainly polymorphonuclear leucocytes. Here and there the epithelium was completely destroyed and the surface was covered by a thick layer of inflammatory cells (Fig. 5 d). In the connective tissue under the damaged epithelium, the vessels showed marked dilatation. Sometimes the necrosis extended down to the underlying connective tissue, which was separated from the healthy connective tissue by a layer of inflammatory cells similar to that seen in the rabbit. New epithelium grew in from the sides and processes containing numerous mitoses extended into the connective tissue (Fig. 6).

The changes in those parts of the mucosa which had been in contact with unpolymerized epoxy resin were characterized by hyperkeratosis which was sometimes very considerable. When the stratum corneum was 20–30 μ thick in areas not covered by the base plate (Fig. 7) (in man it is on the average 13.4 μ (ÖSTLUND, 1953)), it was sometimes as thick as 70–80 μ in areas exposed to the epoxy resin (Fig. 8). The thickness of the hornified layer in the control areas covered with paraffin did not differ from that of uncovered areas.

DISCUSSION

The destructive tissue changes described were strictly limited to those parts of the skin and mucosa which had been in direct contact with diethylene triamine (curing agent). There is thus little doubt that of the three substances, polymerized epoxy resin, non-polymerized resin and curing agent, the last mentioned is by far the most

injurious under the present experimental conditions. This supports earlier observations on persons employed in industries working with epoxy resins.

The use of epoxy resin as a tooth filling material cannot therefore be recommended at present, since the amine curing agent may affect the pulp as well as the gingiva during the long curing time (3-5 hr). The use of epoxy resin for base plates may also be questioned, since it is not known with certainty that the finished plate does not contain residual curing agent.

It is true that neither un-polymerized epoxy resin nor the finished product produced any demonstrable destructive changes, but the time for which the mucosa was exposed to the substance was not long enough to exclude the possibility of later changes. Thickening of the horny layer definitely indicates irritation and the mucosal reaction deserves attention.

From a prosthetic point of view, the thickening of the horny layer was of interest, since earlier investigations (ÖSTLUND, 1958) of the reactions of the mucosa to acrylic base plates regularly showed a reduction in the thickness of this layer. It is hoped that future investigations will demonstrate the eventual effect of this hyperkeratosis.

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FIG. 1. Preparation trays punched from 0.5 mm tin-foil. Holes for sutures.

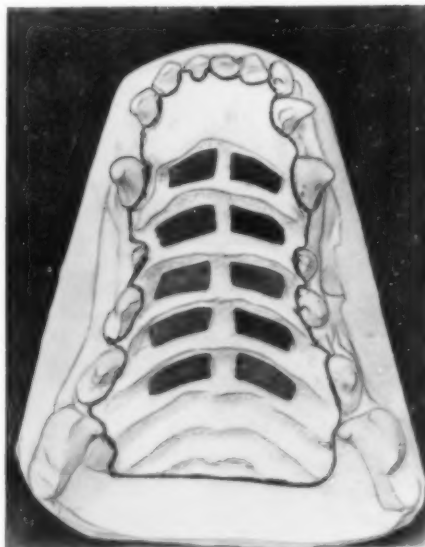


FIG. 2. 1 mm tin-plates for making preparation carrying cavities on base plates.



FIG. 3. Necrotic tissue bordered by a layer of inflammatory cells. $\times 100$.

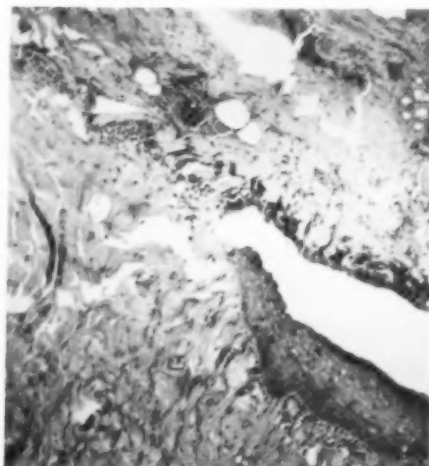


FIG. 4. New epithelium growing in from the outer edge of damaged area. $\times 100$.

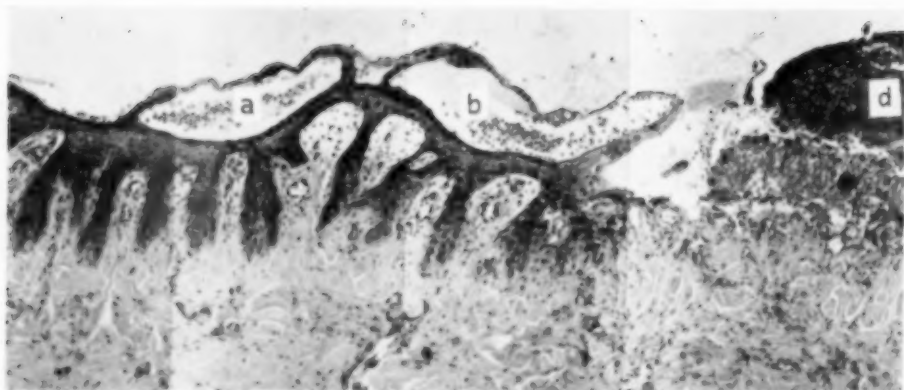


FIG. 5. Epithelium containing vesicular formations (a and b) and here and there inflammatory cells (d). $\times 70$.

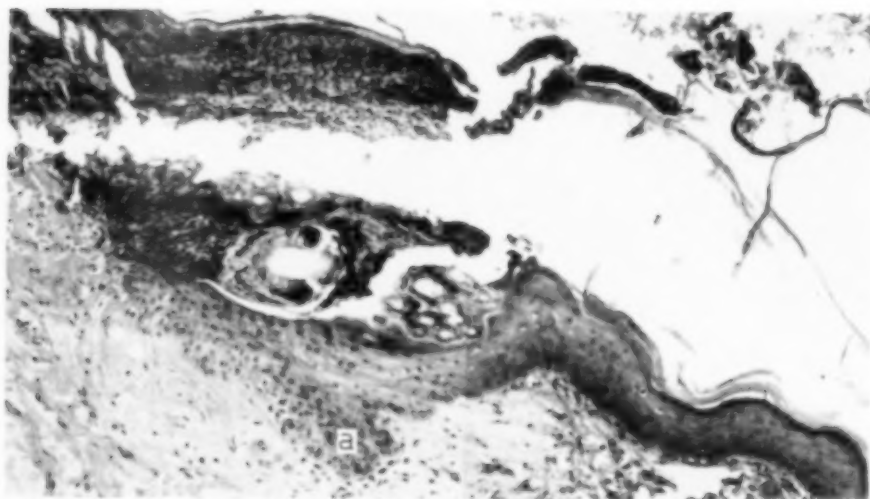


FIG. 6. New epithelium (a) extending into the connective tissue. $\times 150$.



FIG. 7. Stratum corneum not covered by a baseplate. $\times 100$.

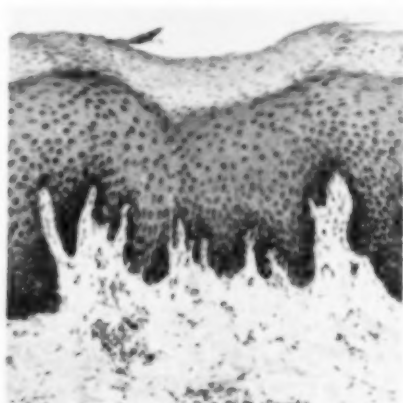


FIG. 8. Stratum corneum in an area exposed to epoxy resin. $\times 100$.

HISTOLOGICAL AND HISTOCHEMICAL STUDIES OF HUMAN TEETH OF THE BRONZE AND STONE AGES

L. I. FALIN

Department of Histology, Moscow Medical Stomatological Institute,
Moscow, U.S.S.R.

Abstract—The microscopic structure of teeth from neolithic and mesolithic sites in the Dniepropetrovsk region showed no changes which could be regarded as evolutionary. Teeth from Bronze Age remains showed a number of defects of development—many interglobular spaces, globular calcification forms in dentine, and well defined growth lines in the enamel. Many teeth were much worn. These phenomena were less obvious in Stone Age teeth. They could not be regarded as the cause of the low susceptibility to caries in these peoples, since similar defects of enamel and dentine development are also found in the teeth of modern man. Post-mortem changes in the form of numerous canals penetrating the dentine were found in neolithic teeth but the mechanism of their formation is unknown. The good state of preservation of the organic matrix of numerous teeth in interments of 4-8 millenia duration is noteworthy. Histochemical behaviour, as well as histological structure of the dentine of decalcified teeth, was well maintained, all the staining and histochemical reactions characteristic of collagen being observed.

IT IS WIDELY ACCEPTED that the prevalence of dental caries in more recent populations is much greater than in ancient times (LUKOMSKY, 1948; KRIKOS, 1935; HOLMER and MAUNSBACH, 1956). It has been suggested that one of the possible reasons for the greater immunity to caries of the teeth of ancient man is their more perfect structure, better calcification and greater resistance to various harmful influences. Studies of the structure of teeth from ancient and prehistoric sources which might confirm or disprove this view are scarce, however, and the inferences drawn are controversial (EULER and WERNER, 1936; SOGNAES, 1955; HELD and BAUD, 1958).

HELD and BAUD noted some deterioration in structure of teeth from modern Swiss in comparison with those examined from neolithic Swiss man and 7th-16th century subjects. On the other hand BRABANT, KLEES and WERELDS (1959), who compared the teeth of present day Belgians with those of 12-16 centuries ago, found no significant differences in structure. As during that period of time the prevalence of caries has risen sharply they concluded that the cause of increase in caries lay in changes in environmental conditions rather than changes in the structure of the teeth.

LUKOMSKY (1948), who studied about 1000 human skulls of the 9th-12th centuries from Eastern Europe, in the territory now known as the Soviet Union, noted a low incidence of caries (average 3.3 per cent). Histologically there was a notable absence of the interglobular dentine which is so common in the teeth of modern man.

Finally, SOGNAES who studied the structure of teeth from ancient and prehistoric man living in various parts of the world (Stone Age of Palestine, predynastic Egypt, Greece of various periods of its history, Norway and Central America) concluded that the existence was widespread of such developmental defects as sharply defined incremental lines in enamel, marked festooning of the enamel-dentine junction and zones of interglobular dentine. He regarded these defects as due to metabolic disturbances during infancy associated with nutritional deficiencies. Of particular interest was the high incidence of defects in the teeth from paleolithic man in Palestine who are regarded as the ancestors of modern man. SOGNAES noted that, in spite of the defective structure of these teeth, the prevalence of caries was very low. This conclusion is based on a study of a relatively small number of teeth (five only from paleolithic Palestine) and therefore need corroboration. The contradictions between the findings of the various workers quoted also indicates the need for further studies in this field.

Moreover, the study of the structure of the teeth of prehistoric man is of more general interest from the standpoint of anthropology and the evolution of man. According to recent views the process of evolution of tissues is closely associated with the evolution of the whole organism (CHLOPIN, 1946; ZAVARZIN, 1945). That is, as evolution of species occurs there are alterations in the structure of their tissues. Teeth, which may survive destruction for many millenia, provide one of the few opportunities to test the validity of this view and to discover whether in this connexion evolutionary changes in man are still occurring or alternatively ceased several millenia ago with the emergence of *Homo sapiens* (BYSTROV, 1957).

Finally, there is interest in the possible survival of organic matter in teeth that have lain in earth for enormous periods of time.

MATERIALS AND METHODS

A total of forty-eight teeth was studied histologically (Table 1).

Bronze Age (2nd and 3rd millenia B.C.). Twenty-seven teeth from eight male and five female skeletons from diggings made by A. P. Trenojkin (1951-1952) in the villages Troizkoje and Akkermin (Zaporogie regions of the Ukrainian S.S.R.).

Neolithic Age (3rd and 4th millenia B.C.). Ten teeth from one male and one female skeleton from diggings made by M. I. Rudinsky (1949-1952) in the village Vovnigi (Dnepropetrovsk region of the Ukrainian S.S.R.).

TABLE I

Age	Types of teeth				Total no. of teeth studied
	Incisors	Canines	Premolars	Molars	
Bronze	7	5	5	10	27
Neolithic	2	2	2	4	10
Mesolithic	—	—	3	8	11

Mesolithic Age (4th and 5th millennia B.C.). Eleven teeth from diggings made by A. D. Stoljar (1953) in the village Vassilievka and by V. N. Danilenko (1952) in the village Voloshskoye (Dnepropetrovsk region of the Ukrainian S.S.R.).

Ground sections were prepared of the majority of the teeth. In view of the fragility of many of them they were embedded in methyl methacrylate in the manner described by SOGNAES (1947). By this means quite thin longitudinal sections of the whole teeth were achieved. Some teeth were decalcified in 5% nitric acid, 5% trichloroacetic acid or in Trilon B at neutral pH. Decalcification showed the presence in these ancient teeth of a well-preserved organic matrix in the dentine. Decalcified teeth of the Bronze and Mesolithic Ages were of cartilaginous consistency and differed but little from those of modern man. Neolithic Age teeth, however, were more fragile. The dentine was dull and yellowish-brown. They became decalcified very speedily, becoming soft and friable, and usually floated on the surface of the decalcifying fluid.

The decalcified teeth were embedded in celloidin and sections were stained with haematoxylin and eosin, van Gieson's picrofuchsin, Heidenhain's azocarmine method, Schmorl's thionin-picric acid technique, Lillie's allochrome method and Foot's silver impregnation. Some sections were stained by the periodate-Schiff technique (SHABADASH, 1947).

In order to obtain data on the prevalence of dental caries in Stone and Bronze Age people, we studied (in collaboration with Dr. V. S. Ivanov) the craniological collection of the Anthropological Institute in Moscow. It was established that caries in men of the Stone and Bronze Ages is observed in less than 0.5 per cent of cases.

RESULTS

Bronze Age

Marked attrition was a notable feature. The height of the crowns of some molars was reduced to 1.7–2.2 mm and in such teeth the occlusal surface was composed largely of secondary dentine consisting of fused laminated rounded nodules containing a few tortuous tubules. In other cases attrition affected only the cusps and was associated with secondary dentine in the horns of the pulp chamber.

The tubule system in all the teeth was normal. Dichotomous branching of tubules was observed (Fig. 1) and in the molar cusps numerous dentinal tubules penetrated the enamel or terminated as enamel spindles. Only in one of the incisors, at the incisive edge, were spindles present.

Numerous areas of interglobular dentine were present in the crowns and in one instance in the root. Irregular interrupted zones of interglobular dentine, single or multiple, and passing obliquely to the outer surface of the dentine (Fig. 2) were common in the crowns of the teeth and less common in the roots.

The enamel-dentine junction of most premolars and molars was either flat or slightly festooned but that of canines and incisors was usually scalloped (Fig. 3).

The structure of the enamel was not notably different from that of modern man. Cross-striations of the enamel rods were distinct and in most instances clearly defined

striae of Retzius were present, especially in the cervical enamel (Fig. 4). In some cases as many as ten-twenty lines of Retzius could be counted in the outer layers of enamel, while few or none were present in the more deeply situated enamel. This would indicate a period of intermittently disturbed enamel formation following an earlier period of more even calcification.

Enamel tufts and enamel lamellae, which are generally believed to consist of uncalcified or less calcified enamel matrix (JASSVOIN, 1936; APPELBAUM, 1948; ORBAN, 1953) and may be particularly susceptible to attack by caries, were present in most molars but not to an extent greater than in the teeth of contemporary man.

In some teeth well-defined irregular yellowish-brown areas extended from the surface appreciably into the thickness of the enamel. Within these areas the outlines of the rods and their cross-striations were more evident. This change in the enamel is probably the result of the action post-mortem of substances dissolved in the soil water.

The structure of the cementum was normal. In cellular cementum, which covered the inner surfaces of roots as well as the root apices, characteristic lacunae were seen.

In decalcified sections the tubules in dentine were easily distinguished and it is of special interest that in many instances Tomes' fibres staining blue with haematoxylin were clearly seen within the dentinal tubules, especially in the dentine nearest the pulp cavity where the fibres were also larger than elsewhere (Fig. 5). Many of the fibres appeared to be broken into wavy fragments. They gave a positive Schiff reaction after preliminary oxidation in potassium periodate, staining bright red or purple. The lacunae of cementum gave a similar positive reaction. Control sections omitting preliminary oxidation were quite colourless.

The matrix of dentine and cementum in the decalcified sections stained bright pink with eosin and with picrofuchsin and bright blue with aniline blue; that is, it gave the staining reactions typical of collagen and with an intensity similar to that given by fresh teeth.

Staining with toluidine blue was very uneven, in some cases with a distinct pink or red-violet metachromasia and in other areas very pale. Tomes' fibres were either deep violet or, rarely, reddish violet.

The interglobular dentine was represented by spaces bounded by the convexities of calcospherites and occasionally contained small discrete calcospherites, but the uncalcified interglobular dentine had not survived. The globular pattern resulting from the fusion of calcospherites that occurs during its calcification was seen throughout the dentine in preparations impregnated with silver by Foot's method (Fig. 6). Particularly large globules were present in the crown in the region of the cusps. It is of interest that in ground sections of the same teeth there was but little interglobular dentine in the crown. According to MEYER (1951) the globular pattern of dentine becomes less evident as age advances owing to further deposition of calcium salts between the globules. In these Bronze Age teeth from adults, however, the globular pattern was very evident and, like interglobular dentine, probably indicates disturbances in the process of calcification, perhaps due to dietary deficiencies. The marked attrition shows that the diet was of a coarse character.

In the inner part of the dentine under the occlusal surfaces in some teeth thirty-forty well-marked regularly-spaced incremental lines were present. These also were indications of disturbances of calcification which from the location in the teeth occurred before eruption (Fig. 7).

Mesolithic Age

Although most of the teeth were from adult or elderly subjects, in contrast to the Bronze Age group only two of the mesolithic specimens showed any attrition, in one molar affecting the cusps only and to a small degree and in the other the cusps were more worn but the enamel was still intact. There were well-marked fissures up to a depth of 0.9–1.0 mm on the occlusal surfaces between the cusps of the molars. As one would expect in the absence of attrition, there was notably less secondary dentine in these teeth than in the Bronze and Neolithic Age groups.

Some of the teeth were affected by post-mortem change in the form of yellowish-brown coloration of the enamel as in the Bronze Age specimens.

Cross-striation of the enamel rods was very evident (Fig. 8) and, especially in the cusps, parazonies and diazones or bands of Schreger due to the wavy course of groups of rods could be seen (Fig. 9). Retzius' lines were marked and formed arches in the enamel over the cusps. Tufts were well-marked in all the molars but enamel lamellae were not so numerous as in the Bronze Age teeth.

Usually Retzius' lines cross the enamel rods at an angle of about 45°. In one mesolithic tooth (a premolar from a 35–40 year old woman) we observed a deviation from this rule. The enamel rods, leaving the enamel-dentine junction in the region of the cervix, turned steeply downwards and then, describing an arch, passed to the enamel surface. Consequently the Retzius' lines crossed the enamel rods almost at a right angle (Fig. 10).

The enamel-dentine junction and number of and distribution of enamel spindles resembled those found in the Bronze Age specimens.

Areas of interglobular dentine were observed in the crowns of all the mesolithic teeth examined. In most cases they were of small dimensions. In two cases only they were found in the dentine of the root. The globular pattern of dentine in several teeth from adult mesolithic men was also seen in the decalcified sections impregnated with silver by Foot's method but in other specimens of similar age calcification of the dentine was more homogeneous.

Similarly to teeth of the Bronze Age the matrix of dentine in the decalcified sections stained bright pink with eosin and with picrofuchsin and bright blue with aniline blue. It gave also a slightly positive periodate-Schiff reaction. Toluidine blue stained the matrix of dentine with a marked pink or red-violet metachromasia. Tomes' fibres were a dark violet, i.e. did not show metachromasia.

Neolithic Age

The teeth showed moderate attrition, especially in the incisors and canine teeth in which at the incisive edge small areas of dentine were exposed. In molars attrition of the enamel was observed mainly on the cusps.

In all teeth examined the peripheral zone of the enamel showed post-mortem changes in the form of deeply pigmented areas. Retzius' lines were less marked in the enamel of incisors and more strongly marked in the enamel of canine teeth, premolars and molars. Enamel spindles were found in only one of the molars. Enamel tufts were common in the molars and premolars. Enamel lamellae were evident in transverse sections of the crowns of the premolars but not in other teeth.

The dentine of neolithic teeth was affected by considerable post-mortem changes similar to those described by SOGNAES (1955) and BRABANT *et al.* (1959) in teeth from fossil man in other parts of the world. These changes consisted of a penetration of the dentine, almost throughout, by thick threads and canals which branched and anastomosed. In places they were so dense that they fused into a common opaque mass (Fig. 11). Some of these formations were slightly yellowish, while others were black. The character of these formations was clearer in decalcified sections, in which the dentine and cementum were penetrated by an enormous number of empty canals which anastomosed and gave the dentine a lace-like appearance (Fig. 12). In ground sections the cavities of these canals were apparently filled with mineral deposits derived from the soil, which are dissolved in the process of decalcification so that their lumina become visible. Thin layers of the remaining dentine, and that between the canals, stained bright pink with eosin or blue by the method of Lillie and gave the other histochemical reactions characteristic of dentine.

DISCUSSION

A number of conclusions may be drawn from this study of a relatively small number of teeth from the Bronze and Stone Ages of 2-6 millenia ago. Firstly, the basic structure of the dental tissues does not differ from that found in modern man. No evidence was found of any differences which might be attributed to evolutionary changes. The period of time involved, however, is perhaps too short for the evolution of histological changes of sufficient magnitude to be detectable and a study of material from man of even more ancient periods would be worthwhile.

The comparative prevalence of developmental defects of enamel and dentine structure in the teeth of Bronze and Stone Age man that this study reveals is in accord with the findings of SOGNAES (1956) and removes any basis for arguing that the low incidence of dental caries in people of those ancient times is due to the better structure of their teeth in comparison with those of modern man.

The prevalence of interglobular dentine and other defects of calcification in the teeth of ancient man may be associated with metabolic disturbances in infancy, dependent on the poor quality of their nutrition.

There is no doubt that the peculiar canals that penetrated the dentine of all the neolithic specimens examined, and some of the Bronze Age and mesolithic also, are post-mortem in origin but the mechanism responsible is not at all clear. SOGNAES described similar canals in human teeth that had lain in the earth for periods ranging from 500 years to several millenia and attributed them to the action of invading fungi. BYSTROV (1956) noted a similar destruction of fossil skeletal elements by fungi. According to SOGNAES there is no definite relationship between the length of burial

and the degree of severity of this post-mortem change. Changes of this character were not observed for instance in the teeth of Egyptians of the predynastic period but were present to a marked extent in 90 per cent of Norwegian material not older than 500 years.

Our observations that the post-mortem changes were well marked in neolithic teeth but not to nearly the same extent in teeth from the Bronze and Mesolithic Ages confirms the need for caution in judging the time of burial from the state of preservation of the teeth. The evidence put forward by SYSSOYEVA (1958) on the changes in the inorganic structure of teeth 10 years after burial and on the formation of deep fissures in the enamel after 40–50 years requires re-examination.

The degree of preservation of organic matter in these ancient teeth is of special interest. It is surprising to find that the histochemical properties of the dentine matrix are still preserved.

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FIG. 1. Dichotomous branching of dentinal tubules in a molar root. (Bronze Age, N 9287). Ground section. $\times 280$.

FIG. 2. Areas of interglobular dentine in the region of the cervix of an incisor. (Bronze Age, N 9195). Ground section. $\times 140$.

FIG. 3. Dentino-enamel junction forming large festoons. Canine tooth. (Bronze Age, N 9273). Ground section. $\times 70$.

FIG. 4. Well-marked Retzius' lines in the enamel of a canine. (Bronze Age, N 9287). Ground section. $\times 70$.

FIG. 5. Tomes' fibres in the internal zone of the dentine of the root. Decalcified section. Haematoxylin and eosin. $\times 280$.

FIG. 6. Globules of dentine and interglobular spaces in the crown. (Bronze Age, N 9200). Decalcified section, Foot's silver impregnation. $\times 70$.

STUDIES OF HUMAN TEETH OF THE BRONZE AND STONE AGES

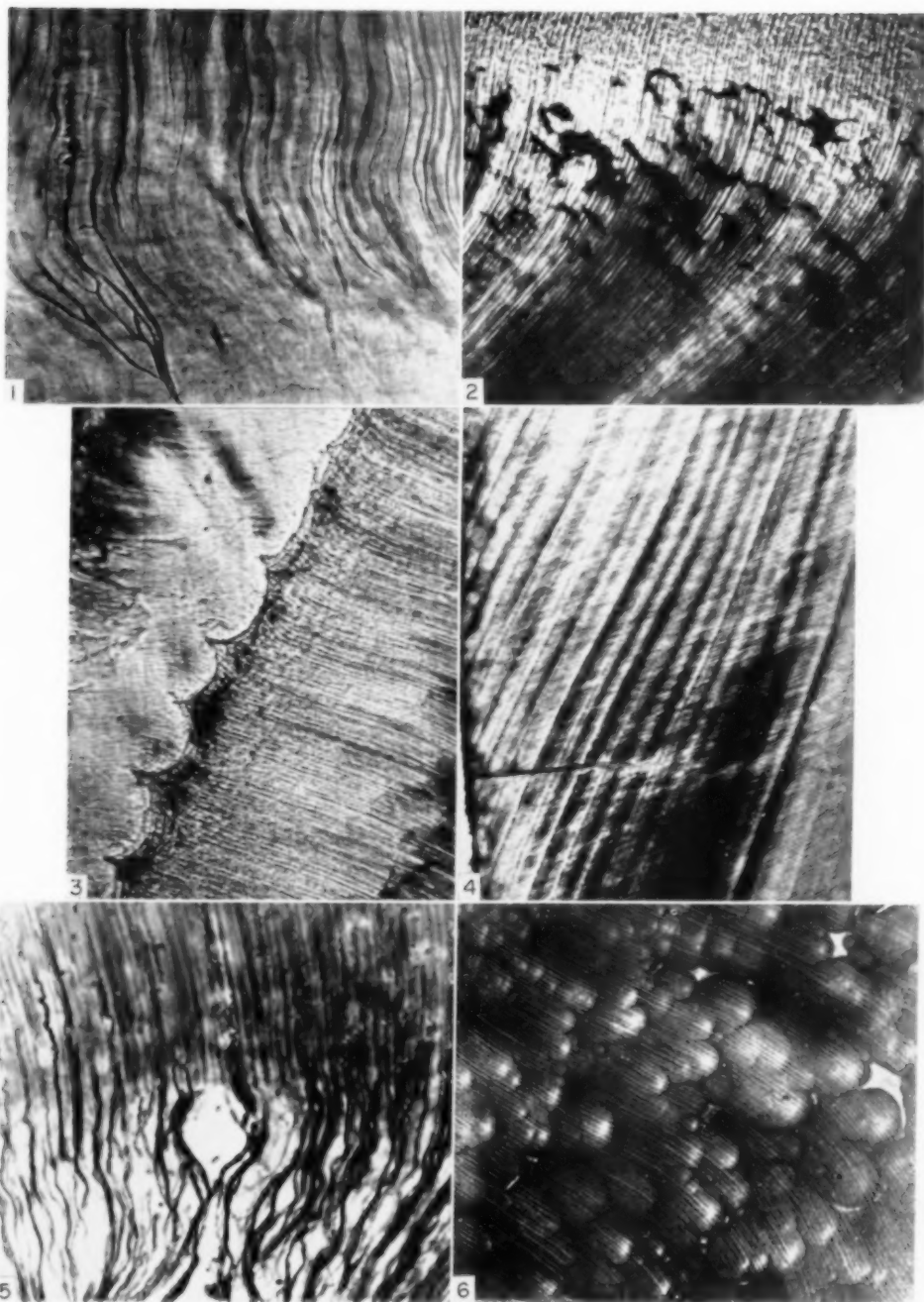


PLATE I

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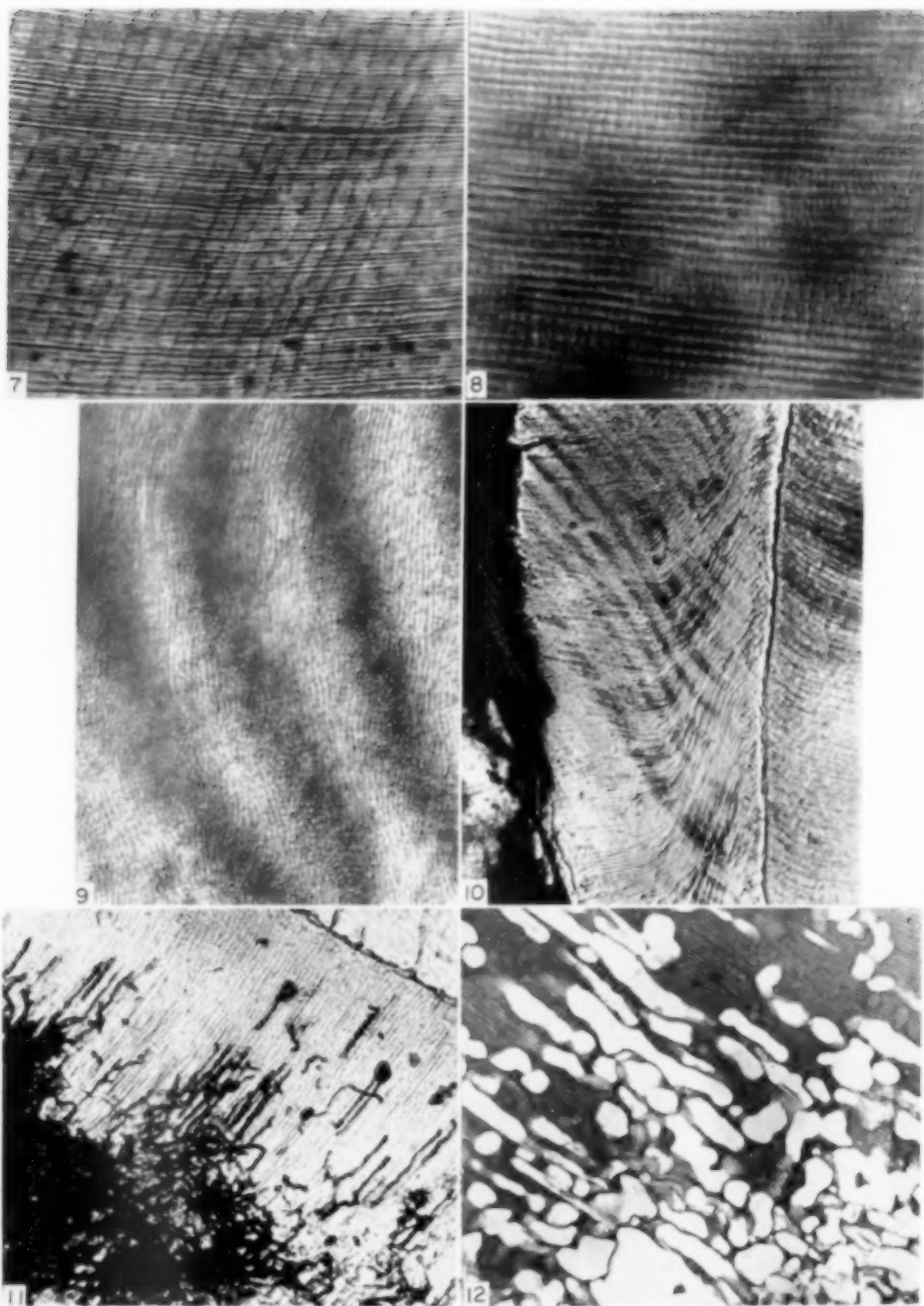


PLATE 2

FIG. 7. Contour lines of Ebner in the dentine of the crown. (Bronze Age, N 9200). Decalcified section, picrofuchsin. $\times 140$.

FIG. 8. Cross-striation of the enamel rods in a mesolithic premolar. Ground section. $\times 280$.

FIG. 9. Parazones and diazones in the enamel of a mesolithic molar. Transmitted light. Ground section. $\times 140$.

FIG. 10. Wavy course of the enamel rods near the cervix. Mesolithic premolar. Ground section. $\times 70$.

FIG. 11. Post-mortem changes in the dentine of a neolithic premolar. Black "threads" piercing the dentine are seen. Ground section. $\times 70$.

FIG. 12. Post-mortem changes in the dentine. Clear canals penetrating the dentine are seen. Decalcified section, Heidenhain's azan. $\times 140$.

THE ARRANGEMENT OF CRYSTALLITES IN ENAMEL PRISMS

D. F. G. POOLE and A. W. BROOKS

The Dental School, University of Bristol

Abstract—A series of X-ray diffraction patterns has been recorded from sections of permanent and deciduous human enamel. The sections were cut in three different planes so that an idea of the spatial arrangement of the crystallites could be found. From any point in the enamel the pattern consisted of arcs of reflection, the variation in crystallite orientation being indicated by the limits of the arcs, and the mean direction of orientation being shown by a line joining the midpoints of the two 002 reflections. In cuspal enamel of longitudinal sections and in enamel cut in the transverse oblique plane, the mean direction of the crystallites was parallel with the direction of the prisms. In enamel on the sides of the crown (lateral enamel) in longitudinal sections, the mean direction of orientation was displaced away from the prism axes towards the cervical margin.

No true "double" orientation of crystallites within the prism complex was demonstrated. This was in agreement with a new interpretation of the optical behaviour of enamel between the crossed polars of a polarizing microscope. There appeared to be a gradual change in the orientation of crystallites from one side of a prism to the other. Such a pattern would account for the features of enamel structure observed here and would agree well with the appearance of enamel as seen with the electron microscope.

Occasionally, diffraction patterns with two sets of arcs were recorded but they were the result of including two differing layers of enamel in the X-ray beam. Specimens producing such an artifact have been used to demonstrate the cause of the asymmetric, distorted reflection patterns which are frequently obtained when working with calcified tissues.

INTRODUCTION

A REVIEW of the classical research into the fine structure of enamel is given by SCHMIDT and KEIL (1958) from which it is evident that crystallites of hydroxyapatite are preferentially arranged within the prisms. Estimations of the dimensions of these crystallites vary considerably (see SCOTT and NYLEN, 1960) but it is fairly certain that normally the crystallites are too small to be seen with an ordinary microscope. Each crystallite is elongated in shape, the direction of elongation being along the crystal *c* axis, or fibre axis as it is commonly termed (THEWLIS, 1940). An enamel prism, therefore, contains a multitude of crystallites, the fibre axes of which tend to be in a definite direction relative to the prism rather than being arranged at random.

Many years ago it was established that the fibre axes of the crystallites are not coincident with the axes of the prisms (VON EBNER, 1890; KEIL, 1936). Later (THEWLIS, 1940) it was established that this deviation of the crystallites was always in the same direction, the outer ends of the crystallites pointing away from the cusp towards the

cervical margin of the tooth. On the other hand, when examined between the crossed polars of a polarizing microscope, the crystallites in the interprismatic substance appeared to have a different orientation from those within the prism (KEIL, 1936). A "double" orientation of crystallites in enamel therefore appeared to exist, one group of crystallites being within the prism and deviating from the prism axis by about 5° , the other group being in the interprismatic substances and deviating from the prism axis by as much as 40° . What appeared to be confirmation of this suggested arrangement was obtained from X-ray diffraction studies (THEWLIS, 1940). With sections of deciduous enamel, diffraction patterns were frequently produced showing two sets of arcs separated by $30\text{--}35^\circ$ and indicating the existence of two groups of crystallites. Similar diffraction patterns have been published more recently (HAMMARLUND-ESSLER, 1958; CARLSTROM, 1960). The same technique of diffraction was used in a slightly different way by TRAUTZ *et al.* (1953), who studied the distortion of the X-ray reflections in order to determine any tilting of the crystallites from the plane of the section towards the incident X-ray beam.

In many of these investigations, the area of enamel under scrutiny was not always clearly specified and attempts to determine possible variations from one area of enamel to another were made by LYON and DARLING (1957) on permanent teeth and CRABB (1958) on deciduous teeth. Sections of such teeth were examined between crossed polars, the direction of the prism and the extinction position being noted and compared to give an indication of the mean crystallite deviation relative to prism direction at any given point. Broadly speaking, similar features were found in both permanent and deciduous enamel. Along a line perpendicular to the amelodentinal junction, the deviation of crystallite fibre axes tended to be less in the middle region of the enamel than in either the inner or the outer regions. Furthermore, deviation increased along the enamel from cusp to cervical margin.

The work to be described here began as an attempt to learn more about the relative distribution of the two sets of 5° and 40° crystallites, as well as their true spatial relationships. For such an investigation X-ray diffraction studies were considered desirable since these could readily detect the presence of two groups of crystallites and also demonstrate deviation and tilt of the crystallites relative to the prism direction. However, constant reference to the appearance of the enamel under consideration between crossed polars was also necessary.

MATERIAL AND METHODS

A considerable amount of material was generously made available by Dr. D. G. LYON and Dr. H. S. M. CRABB of the University Dental School, Bristol. This included ground sections of human permanent teeth cut in three different planes (Fig. 1) and buccolingual longitudinal sections of deciduous teeth. Initially whole sections were used but later it became necessary to examine splinters of enamel, so that diffraction patterns of a particular spot could be taken with reference to different planes of the prisms. Such splinters were obtained from whole sections by cutting through the dentine along a line parallel with the prism direction in the enamel (BB' , Fig. 1). When the cut reached the amelodentinal junction, the enamel tended

to fracture along the line of cut already established. In this way splinters were obtained running from the amelodentinal junction to the surface, and about 15 prisms wide in each cross-sectional dimension. Part of the dentine remained attached to the enamel splinters.

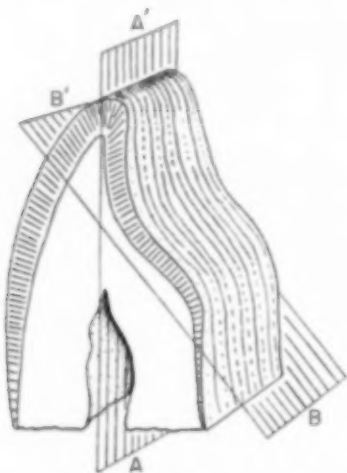


FIG. 1. Diagram of the crown of a human incisor to demonstrate the three planes used in the preparation of sections. The appearance of a section cut in the buccolingual longitudinal plane is shown and the approximate direction of the prisms in the enamel is indicated by the hatching. The mesiodistal longitudinal plane (AA') and the transverse oblique plane (BB') are also shown and it will be noticed that, in each case, prisms tend to lie in the plane of sectioning.

The diffraction unit employed was a Phillips type 11704/23 having a sealed tube, copper target, mica-beryllium windows and nickel filters. A flat plate camera, type 1030, with a collimator width of 0.5 mm was normally used. In some studies the collimator size was reduced to 0.2 mm, thus limiting the area of diffracting enamel to one sixth of that with the standard collimator.

The specimen plate holder was permanently fixed in such a position that, when in place, the edges of the plate were parallel with those of the film-holder. The section was mounted on the specimen plate under a microscope so that prisms could be accurately aligned with the vertical axis of the plate. After positioning in this way, Farrant's medium was used to attach the specimen to the plate. Thus, when the plate was slotted into its holder, the prisms were automatically aligned with the vertical axis of the film-holder.

For the examination of enamel splinters, the dentine portion was cemented with gum acacia to the end of a pin (Fig. 2t) mounted in a small cork. The cork was embedded at the top of a column of "Plasticine" which was then moulded until the splinter stood vertically across the collimator exit. A marker on the cork indicated the original plane of sectioning and, by rotating about the axis of the pin, the direction

of the incident beam relative to the splinter could be altered. The direction of the prisms relative to the axis of the splinter was later determined under the microscope.

Approximately 100 diffraction patterns were recorded and examined during the investigation.

X-RAY DIFFRACTION STUDIES

Permanent enamel

The sections used in this part of the investigation were prepared principally from central incisors and from premolars. Three planes of sectioning were chosen in each of which the prisms lie largely in the plane of the section. The section planes are illustrated in Fig. 1 and may be referred to as the buccolingual and mesiodistal longitudinal planes, and the transverse oblique plane. When the results from these three planes are considered, an idea of the spatial arrangement of the crystallites is obtained. In order to keep sources of error to a minimum, only sections in which much of the enamel contained straight, parallel prisms were used. For the same reason attention was paid to the outer two-thirds of the thickness of enamel which tends to show a more regular prism alignment than the inner third. Finally, efforts were made to ensure that in the area of enamel to be examined, prisms were straight and parallel throughout the thickness of the section.

Mesiodistal sections. In such sections an extensive region of cuspal enamel is present (Fig. 1). A diffraction pattern from the central cuspal area is shown in Fig. 2 (a), the 002 reflections giving a clear indication of the orientation of crystallites in this region. The arcs of reflection are short and intense, indicating that the variation of the crystallite orientation is about 15–20°. Furthermore, a line joining the mid-points of the two 002 reflections is very nearly parallel with the direction of the prisms. Very similar patterns were obtained along the whole of the cuspal area, although a slight increase in the arc of spread occurred towards both mesial and distal extremes. This means that in the cuspal region of mesiodistal sections, the crystallites of hydroxyapatite show a high degree of preferred orientation, tending to lie parallel with the direction of the prisms.

However, at the mesial and distal incisal angles of the section the pattern begins to change. As seen in Fig. 2 (b) the 002 reflections from such areas are more extended and a line joining the centres of the arcs is no longer parallel with the prism direction. Thus, at the upper, cuspal extremes of lateral enamel there is a tendency for the variation in crystallite orientation to be greater as well as a tendency for the mean of this variation to be displaced from the direction of the prism towards the cervical margin of the tooth. It should be noted here that the term "lateral enamel" is used throughout this paper to indicate any enamel other than cuspal enamel seen in longitudinal sections. Fig. 2 (c) is a pattern from enamel near to the cervical margin showing this tendency even more markedly. The arcs are no longer of symmetrical intensity because, although the total spread of crystallites is still about 40°, the majority are towards one end of the arc and now deviate considerably from the direction of the prisms. A series of such patterns along the whole of the lateral enamel suggests that from the cusp to the cervical margin there is a tendency for the crystallites to deviate more and more from the prism axes.

Buccolingual sections. Results here were similar to those obtained with mesiodistal sections. Only a limited area of cuspal enamel is present in buccolingual sections and over the tip of the cusp the prisms tend to have a radial disposition (Fig. 1). The 002 reflections were rather more extended than in mediiodistal sections but again the crystallites are mainly arranged parallel with the direction of the prisms. Down the buccal surface deviation of crystallites again occurred. In some cases deviation increased gradually from cusp to cervical margin but in others it remained remarkably constant along most of the buccal enamel (Figs. 2m,n), only increasing in the immediate vicinity of the cervical margin (Figs. 2o,p).

Transverse oblique sections. A typical pattern from such sections is given in Fig. 2 (d) and it is almost identical with patterns from cuspal enamel (Fig. 2a), which means that enamel viewed in this plane shows little crystallite deviation. The mean direction of the crystallites is parallel with the prism direction, crystallites being spread in a short arc which is symmetrical about this line.

From these results, the indications are that appreciable crystallite deviation away from the prism axis towards the cervical margin occurs only in lateral enamel viewed in planes radial to the longitudinal axis of the tooth. In cuspal enamel, or enamel viewed in transverse oblique planes, crystallite deviation is relatively slight.

Such conclusions agree well with those drawn from the examination of the same sections between crossed polars (LYON and DARLING, 1957). However, the most surprising result of the investigation was the almost complete lack of indication of any "double" orientation of the crystallites. Distorted, uneven arcs were frequently obtained (Figs. 2c,m,n) but at this stage of the work no pattern showing two separate sets of reflections had been found. Because of this, attention was turned to deciduous enamel in which "double" orientation was originally described.

Deciduous enamel

Sections of deciduous incisors and molars were used, cut in the buccolingual longitudinal plane. In addition splinters of enamel, prepared as described earlier, were also examined. By means of such splinters an area of enamel could first of all be examined in the original plane of the section and then, by rotating the splinter through 90° , patterns of the same enamel in the transverse oblique plane could also be recorded. As with the sections of permanent teeth, attention was paid mainly to the outer enamel.

Initially a series of buccolingual sections was examined. Some of these, when later examined in splinter form, produced single sets of arcs in both longitudinal and transverse oblique planes. Such patterns were so like those described in permanent enamel that a further description will not be necessary. However, the majority of sections produced diffraction patterns similar to the one illustrated in Fig. 2 (e), with clearly defined double arcs such as were described by THEWLIS (1940). Obviously, two sets of crystallites were being recorded, one with an apparently low mean deviation of approximately 5° , the other with an apparently high mean deviation of approximately 40° . Work was continued with splinters from such sections in an attempt to determine the true spatial relationships of the two groups. The inter-

pretation of the patterns was not easy, but an example will be described in detail since it helps to explain some of the anomalies often seen in X-ray patterns.

Fig. 2 (i) is the pattern given by an enamel fragment arranged, relative to the X-ray beam, as it would have been in the original buccolingual plane of the section. The pattern is therefore the same as that given by the original section. Figs. 2 (j,k,l) are, respectively, the patterns given by the same enamel splinter rotated about its long axis into positions 90° , 180° and 270° from the original position. The patterns in the 90° and 270° positions are of the enamel in the transverse oblique plane. It is always necessary to bear in mind that the splinter itself was mounted vertically in the camera and that only very rarely was the axis of the splinter parallel with the prisms. Thus, in Fig. 2 (i), the prism direction was very approximately parallel with the set of crystallites indicated by the left hand upper arc.

It will be noted that in Fig. 2 (e) the upper arcs are more intense than the lower, and in Fig. 2 (i) the lower arcs are almost invisible. This is because the fibre axes of the crystallites not only deviate from the prism direction but are also tilted out of the plane of the section. As explained by TRAUTZ *et al.* (1953), the upper ends of the crystallites are tilted towards the incident X-ray beam, and almost all the patterns given in Fig. 2 show some greater or lesser degree of tilt. Fig. 3 (a) is a diagrammatic representation of two groups of crystallites deviating to either side of the vertical but tilted obliquely in the same direction, upper ends forwards. The resulting 002 reflections are also represented and it will be noted that the upper arcs are more intense than the lower. If the specimen containing these two groups of crystallites is rotated about the vertical through 90° , the original angles of deviation become angles of tilt, and the original angles of tilt become angles of deviation. In

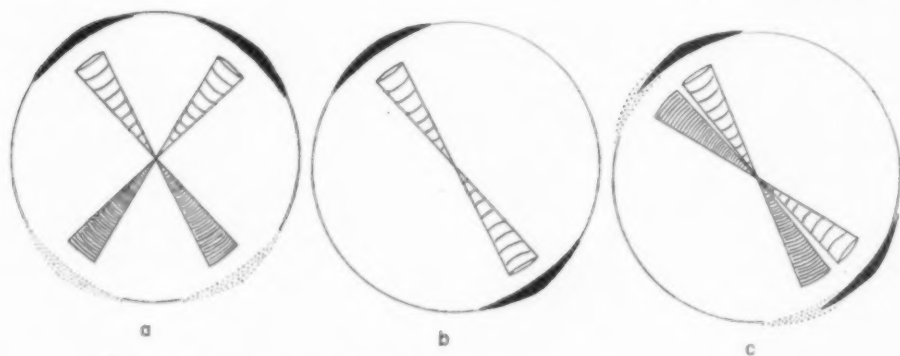


FIG. 3. Diagram showing the effect of crystallite deviation and tilt upon the resulting X-ray diffraction patterns.

(a) Two groups of crystallites with opposite deviation from the vertical but tilted in the same plane. The upper ends of the crystallites, shown by the light hatching, are tilted forwards towards the incident X-ray beam.

(b) The same piece of enamel turned through 90° . The two groups are now superimposed although one group tilts forward and the other backwards.

(c) If the rotation is not quite 90° , complete superimposition does not occur and the resulting diffraction pattern will show apparently distorted arcs.

Fig. 3 (a) the angle of tilt is the same for each group so that in the 90° position (Fig. 3b) they become exactly superimposed. But since the original deviations were to either side of the vertical, in the 90° position one group of crystallites will tilt forwards and the other backwards. The final diffraction pattern would show only a single set of arcs deviating from the vertical, but upper and lower arcs of equal intensity (Fig. 3b).

If, however, the rotation of the specimen had been slightly less than 90° , the two sets of crystallites would not become exactly superimposed and an apparently distorted pattern would result (Fig. 3c). Distortion would also occur if, in the original plane, the two groups of crystallites were tilted in the same direction but by different amounts, for again they would not become superimposed in the 90° position. This is the explanation of the distorted pattern in Fig. 2 (j), and such patterns may well be further distorted by other factors such as the two groups being of unequal size, or deviation in the original plane not being symmetrical about the vertical. Distorted patterns are frequently obtained when working with calcified tissues and are occasionally seen in print (POOLE, 1956; LITTLE, 1960). Of course, even single groups of crystallites can produce similar distortions if, across the arc of spread, variation in tilt occurred from one extreme to the other. The patterns given in Figs. 2 (k,l) are, as might be anticipated, inverted forms of Figs. 2 (i,j) respectively and need not be described in more detail. However, further reference to this crystallite complex will be made below.

As yet no reference has been made to the sites of the two groups of crystallites within the enamel. In the past it has been suggested that the crystallites producing the almost vertically placed arc (Fig. 2e) are located within the prism, whereas the arc widely displaced from the vertical is mainly due to crystallites in the interprismatic substance (THEWLIS, 1940).

During the handling and dissecting of deciduous sections it was noticed that, between crossed polars, a narrow zone of the outermost enamel tended to be differentiated from the underlying main part of the enamel. Since these sections were dry when handled, the optical features were usually confused because of strong light refraction by air-filled spaces. Reference was therefore made to sections which had been cleared and mounted in Canada balsam. Some seventy or more sections were examined between crossed polars, two-thirds of which showed a superficial zone with an extinction position quite different from that of the underlying enamel (Fig. 2u). Undoubtedly such a region would be recorded in diffraction patterns of the outer part of the enamel and it was necessary to discover how much of the patterns described above might be due to this zone. The zone, on an average, was 0.1–0.2 mm wide, so that it was not possible to record its pattern alone with the standard 0.5 mm collimator. A collimator made from drawn metal tubing with an average bore of 0.2 mm was therefore substituted and a number of sections subsequently examined. For each section a series of patterns was taken along a line running from middle enamel to the surface and such a series is illustrated in Figs. 2 (f,g,h). The middle enamel shows single arcs near to the vertical (Fig. 2f) and no double orientation can exist here. Outer enamel, also, shows only single arcs which are

displaced widely from the vertical (Fig. 2h) and which indicate the absence of double orientation here as well. However, at the junction between the two zones, with some of each zone included in the X-ray beam, a "double" pattern is established (Fig. 2g). Thus, it is clear that the "double" patterns recorded earlier in this work are artifacts caused by the inclusion of two differing enamel layers in the X-ray beam. Very recently "double" patterns have also been obtained with permanent enamel. These patterns can also be shown to be due to similar causes.

Finally, some reference must be made to the nature of the superficial zone observed in deciduous enamel. In the sections examined, the prisms tended to run parallel and straight towards the surface. Near to the surface the prism boundaries become less distinct but where prisms can be seen they appear to bend abruptly. Thus, the crystallites in this outer layer, still following the direction of the prism, lie in different directions relative to the crystallites in the underlying parts of the prism. Referring back to Figs. 2 (i) and 2 (j), it will be recalled that, whereas in the buccolingual plane two sets of arcs are recorded, in the transverse oblique plane these arcs are superimposed, though not completely. When the inaccuracies in the preparation of the specimens are taken into consideration, it can be shown that the bending of the prisms at the surface is not evident in the transverse oblique planes, and the outer zone is therefore not readily demonstrable in diffraction patterns of the outer third of the enamel.

The conclusions to be drawn from these results are that at any point in enamel the crystallites vary in their directions of orientation. The mean angle of deviation relative to the prism axes varies from 0° in cuspal enamel and enamel of transverse oblique sections to $20-30^\circ$ in lateral enamel of longitudinal sections. Two sets of crystallites do not appear to be a normal feature of prism structure as far as can be demonstrated by means of X-ray diffraction.

DISCUSSION

At first sight the conclusions outlined above conflict with those drawn by earlier workers using similar techniques. However, a scrutiny of the results of THEWLIS (1940) reveals that in many cases patterns of only single arcs were obtained and, furthermore, THEWLIS states that on moving from the amelodentinal junction to the surface the pattern changes from one of single crystallite orientation to one of double orientation. This is precisely what was observed in the present investigation and has been found to be due to an artifact. Thus the results are similar in the two cases, but the extra evidence presented here demands a new interpretation.

The presence of a single preferred orientation of crystallites as shown by X-ray diffraction also conflicts with descriptions of enamel structure based on the appearance of enamel between the crossed polars of a polarizing microscope. It has been observed that if the prisms in a section are rotated towards the extinction position there is no uniform black-out and commonly two areas with differing extinction positions can be seen in each prism (KEIL, 1936; SCHMIDT and KEIL, 1958). The conclusion was that, within the prism, crystallites deviate only slightly from the prism axis and black out when prisms are approximately parallel with the vibration direction of

one of the polars. On the other hand, crystallites in the interprismatic substance tend to deviate significantly from the direction of the prism axes and become extinct only when the prism axes are at an appreciable angle to the vibration direction. There can be no doubt that within the prism complex a variation in extinction position does occur. But again, the important problem to be solved is the exact sites of the differing extinction positions. Performing the same operations on sections from the three planes described above leads to several interesting conclusions.

In cuspal enamel of longitudinal sections or in the enamel of transverse oblique sections it is only rarely possible to demonstrate the two extinction positions described by KEIL. It is more commonly found that, although the individual prisms do not black out completely uniformly, each part of a prism will have blacked out within a very short arc of rotation ($5-10^\circ$) symmetrically placed about the vibration direction. Thus if the prisms are aligned approximately parallel with a vibration direction a pattern of irregular lines of extinction masks the basic prism pattern and it is no longer possible to distinguish one side of a prism from another. Such a result would be expected from the X-ray diffraction patterns of these areas of enamel (Figs. 2a,d).

Nevertheless, the pattern described by KEIL (1936) is to be found along the whole of the lateral enamel in buccolingual or mesiodistal sections, and such areas have here been examined minutely. Figs. 2 (q,r,s) show the different appearances of such an area as the prisms are rotated from the 45° position towards and past the vibration direction of one of the polars. In the 45° position (Fig. 2q) the prism boundaries are seen clearly and the whole of each prism is illuminated. As the vibration direction is approached (Fig. 2r) extinction areas begin to appear and a careful examination reveals that each extinction area is to the same side of a prism boundary. At or just past the vibration direction the whole of one side of each prism is dark and the other side light. The dark side is that nearest the cusp, the bright side that nearest the cervical margin. This means that within each prism the crystallites on the cuspal side tend to lie fairly close to the prism axis, whereas those on the cervical side tend to deviate considerably. On rotating even farther past the vibration direction, the cuspal side gradually lights up and the cervical side darkens (Fig. 2s). Thus the two positions of extinction occur within the self-same prism unit and do not represent prism as opposed to interprismatic substance. The enamel shown in Figs. 2 (q,r,s) is not altogether typical in so far as the region towards the cervical side of a prism, with highly deviating crystallites, is rather wide. More typically it appears to extend over approximately one quarter of the total width of a prism and is much narrower than the cuspal region. It may well be that this difference in width of the two zones has in the past been wrongly believed to represent thicker prisms separated by thinner interprismatic zones. The actual prism boundaries (Figs. 2q,r,s) seem to have little or no birefringence, a fact already recorded by GUSTAFSON (1945) who therefore argued that the area with highly deviating crystallites must be prism sheath. He did not, apparently, note that such zones were limited to the cervical sides of the prisms.

From the examination of very many sections it is evident that there is usually no sharp boundary between cervical and cuspal sides of a prism, but rather a gradation from one to the other. As prisms are rotated past a vibration direction, a black line

of extinction, often accurately parallel with the prism boundaries, passes across each prism indicating a gradual change in crystallite orientation from one side of a prism to the other. The angle of deviation from prism axis gradually increases from the cuspal side to the cervical. In other words, there is a gradual change in the crystallite orientation across the prism and this is just the kind of arrangement required to produce the X-ray diffraction patterns shown in Figs. 2 (b,c). Intensification of the arc at any point would be due to an aggregation of crystallites greater in one direction than in others.

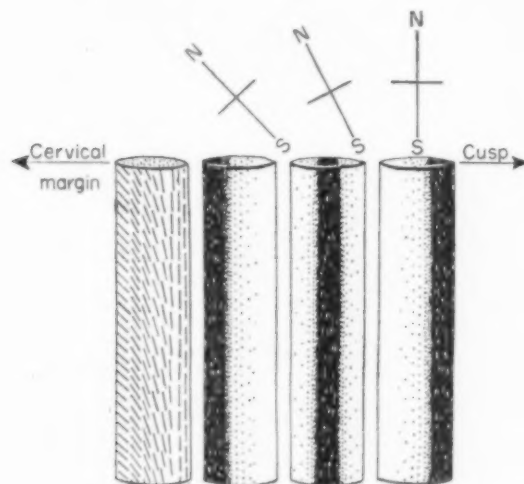


FIG. 4. Diagram to illustrate the arrangement of crystallites in a prism as seen in longitudinal sections of lateral enamel, and also the appearance of this prism in different positions relative to the vibration directions of crossed polars. Extinction areas are shown in black.

When all of these observations are considered together, a scheme of crystallite arrangement within the prisms can be deduced. Lateral enamel, cut in longitudinal planes of the tooth, will show a pattern such as that drawn in Fig. 4. Crystallites on the cuspal sides of the prism are almost parallel with prism axis, those cervically deviate by $20-45^\circ$, whilst between these two extremes is a gradual transition from one to the other. The appearance of prisms with such a crystallite arrangement when seen between crossed polars is also given. In a plane perpendicular to that shown in Fig. 4 (i.e. in the transverse oblique plane), a different arrangement is seen (Fig. 5), again based on the appearance between crossed polars. Fig. 5 attempts to show the complete spatial arrangement of crystallites in lateral enamel and it will be noted that the cross-sectional appearance of the prisms is represented as incomplete rings, the open ends towards the cervical margin. This pattern is frequently observed in ground sections as well as decalcified and stained preparations. Cuspal

enamel would show no appreciable crystallite deviation in any plane. Such a scheme as this will account for all the diffraction patterns and polarized light appearances described here.

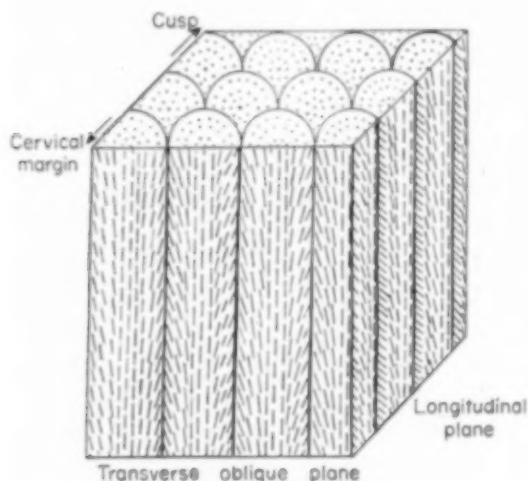


FIG. 5. Composite drawing of lateral enamel showing the crystallite orientation within the prisms.

This suggested crystallite arrangement agrees remarkably well with that actually "seen" with the electron microscope (e.g. HELMCKE, 1955, 1960; LENZ, 1958; FRANK and SOGNAES, 1960). In the latter investigations the plane of sectioning is not always clearly defined but, nevertheless, examination of the various electron microscope photographs which have appeared in print will show most of the different patterns deduced above by indirect means.

It is regretted that no conclusions can be reached here concerning the nature of the so-called interprismatic material which, if it exists at all, appears to separate one prism from another. From decalcified, stained, histological preparations there is no doubt that around the periphery of each prism is a zone with a relatively high organic content. Whether or not this should be regarded as prism sheath or interprismatic substance, whether either of these should be regarded as separate entities or whether again this is a function of graded structure from without the prism to within is still a matter for conjecture. Even recent electron microscope studies fail to solve the problem. Thus, such studies have led to the denial of the existence of interprismatic substance (WATSON and AVERY, 1954; LENZ, 1958; QUIGLEY, 1959), as well as to confirmation of its existence (FRANK, 1959; SCOTT and NYLEN, 1960) and even greater confusion arises from the conclusions of FRANK and SOGNAES (1960). According to the latter, interprismatic substance is present in human enamel but neither it nor prism sheaths can be identified in rat enamel. Yet prisms can be seen in the middle region of rat enamel with both optical and electron microscopes, so

the question immediately arises as to what differentiates these prisms from each other. It is an important question since, according to the same authors, in inner and outer rat enamel crystallites continue to run in the same direction as they did in the middle enamel, yet prisms are not apparent in these two other regions.

However, this conflict could provide clues which, together with work proceeding in this department, may result in a theory which would account for all the real and apparent features of enamel structure.

Acknowledgements—This research was carried out whilst the authors were in receipt of research grants from the Medical Research Council, to whom grateful thanks are offered. We should also like to thank Professor A. I. DARLING, of the University of Bristol Dental School, for initiating and encouraging this work, as well as Dr. D. G. LYON and Dr. H. S. M. CRABB, of the same department, for providing material.

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FIG. 2. (a-p) Diffraction patterns recorded from human enamel at certain points in three different planes.

- (a) Mid-cuspal permanent enamel, mesiodistal section. Collimator 0.5 mm.
- (b) Lateral permanent enamel, mesiodistal section. Collimator 0.5 mm.
- (c) Lateral permanent enamel, at cervical margin, mesiodistal section. Collimator 0.5 mm.
- (d) Permanent enamel, transverse oblique section. Collimator 0.5 mm.
- (e) Outer half deciduous enamel, buccolingual section. Collimator 0.5 mm.
- (f) Middle region deciduous enamel, buccolingual section. Collimator 0.2 mm.
- (g) Junction between superficial and underlying zones of deciduous enamel, buccolingual section. Collimator 0.2 mm.
- (h) Superficial zone deciduous enamel, buccolingual section. Collimator 0.2 mm.
- (i) Deciduous enamel splinter, buccolingual plane (0° position). Collimator 0.5 mm.
- (j) The same enamel splinter rotated 90° to record the transverse oblique plane. Collimator 0.5 mm.
- (k) The same enamel splinter rotated 180°. Collimator 0.5 mm.
- (l) The same enamel splinter rotated 270°. Collimator 0.5 mm.
- (m) Cuspal extreme of lateral permanent enamel, buccolingual section. Collimator 0.5 mm.
- (n) Lateral permanent enamel approaching cervical area, buccolingual section. Collimator 0.5 mm.
- (o) Lateral permanent enamel immediately adjacent to cervical margin, buccolingual section. Collimator 0.5 mm.
- (p) Lateral permanent enamel, at cervical margin, buccolingual section. Collimator 0.5 mm.
- (q-s) Successive appearances of the same area of lateral permanent enamel in a buccolingual section as seen between crossed polars when the prisms are rotated from the position of maximum illumination towards and past the vertical vibration direction. The cusp is to the left and the cervical margin to the right.
- (q) When near to the position of maximum illumination, the prisms are uniformly bright and their boundaries are clearly visible.
- (r) On rotating the prisms near to the vertical vibration direction the prism boundaries are still seen as thin, black lines. In addition to this two distinct areas are apparent within each prism. The left hand side of each prism is becoming dark, but the right hand side remains bright.
- (s) With the prisms rotated well past the vibration direction the left side of each prism is now bright and the right side dark.
- (t) A splinter preparation attached by gum acacia to the point of a pin.
- (u) The appearance of outer deciduous enamel in a buccolingual section between crossed polars. The superficial zone remains bright when the underlying enamel is blacked out, indicating that these adjacent areas have quite different extinction positions.

THE ARRANGEMENT OF CRYSTALLITES IN ENAMEL PRISMS

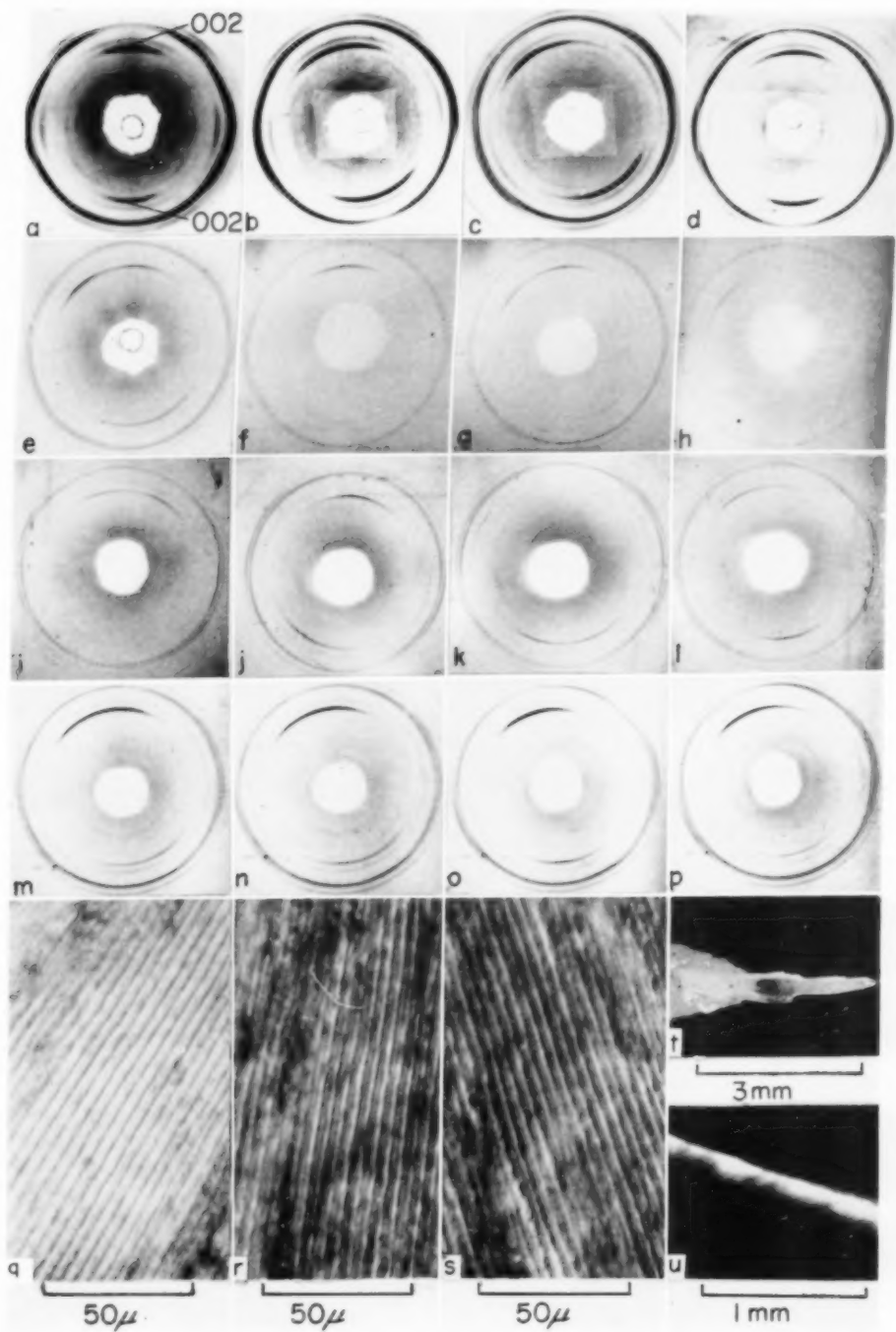


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THE EFFECT OF THYROID FUNCTION ON SUTURAL OSTEOGENESIS IN THE RAT

C. J. BURSTONE and W. G. SHAFER

Departments of Orthodontics and Oral Pathology, School of Dentistry,
Indiana University, Indianapolis, Indiana, U.S.A.

Abstract—A method for artificially expanding the premaxillary suture of the rat was applied to investigation of the effects of thyroid dysfunction on osteogenesis. Three groups, hypothyroid, hyperthyroid and control, were compared as to sutural activity. Variation was evident in the degree of separation at the premaxillary suture. However, no consistent differences among groups were found. No differences were noted in the orientation of the collagen fibres or the vascularity of the connective tissue between experimental and control groups. Propylthiouracil-treated animals showed a more profound tissue reaction at the site of suture separation than either control or hyperthyroid animals. The increased sutural activity associated with the hypothyroid groups was characterized by a greater thickness of osteoid along the inner suture faces, more prominent osteophyte formation, greater osteoblastic activity and an increased cellularity in the connective tissue between the two plates of bone.

CLINICAL and animal investigations have failed to define clearly the role of the thyroid gland in osteogenesis, although there is ample evidence that a relationship does exist.

SIMPSON, ASLING and EVANS (1950) have shown that thyroxin primarily acts to accelerate skeletal maturation and that it has no direct growth promoting effect. These conclusions are based on endochondral growth occurring within the epiphyseal plate of long bones. RAY *et al.* (1950) also found that administration of thyroxin to hypophysectomized rats prevented the arrest of endochondral ossification which normally occurs in untreated hypophysectomized animals. However, the relationship of the thyroid to non-endochondral osteogenesis has not been demonstrated.

Some information is available from the study of patients with endocrine imbalances involving the thyroid gland. Radiographic examination of the hyperthyroid patient frequently demonstrates generalized osteoporosis. An increased radiolucency combined with an increase in calcium and phosphorus excretion in these patients, as reported by AUB *et al.* (1929), suggests that active bone resorption is occurring.

A method for initiating osteogenic changes by mechanically inducing stress in the premaxillary suture of the rat has been previously described by BURSTONE and SHAFER (1959). In applying this artificial sutural expansion technique, the present study had as its aim the relating of thyroid activity to osteogenesis.

METHOD

The premaxillary suture of the rat was expanded utilizing the force produced by deformed $\frac{1}{8}$ in. gum rubber wedges. One hundred male albino rats, 2 months of age, were divided into three series (Table I).

TABLE I. SUTURAL OSTEOGENESIS—EXPERIMENTAL PLAN

Series	Group	No. of animals	Dosage	Dosage initiated	Days of sutural expansion
I	A—Thyroxin	6	100 μ g daily	At time of insertion of appliance	6
	B—Thiouracil	6	0.1% in diet	9 days before insertion of appliance	6
	C—Control	6	—	—	6
II	A—Thyroxin	15	1st week, 25 μ g 2nd week, 50 μ g 50 μ g, thereafter	4 weeks before insertion of appliance	5
	B—Thiouracil	20	0.1% in diet	4 weeks before insertion of appliance	5
	C—Control	15	—	—	5
III	A—Thyroxin	13	100 μ g	2 weeks before insertion of appliance	5
	B—Thiouracil	11	0.1% in diet	2 weeks before insertion of appliance	5
	C—Control	8	—	—	5

In order to produce hypothyroidism, propylthiouracil (0.1 per cent) was introduced into the diet (Stock diet, Hoosier Mineral Feed Company, Greenwood, Indiana) from 9 to 28 days prior to the insertion of the appliance and was maintained during the time of sutural expansion. The histologic appearance of the thyroid was used to verify the presence of hypothyroidism. The hyperthyroid groups were given between 25 and 100 μ g of thyroxin daily (subcutaneously). The thyroxin injections were initiated 0–28 days before the insertion of the appliance and were continued throughout the experiment. 5–6 days following the placing of the sutural expansion appliance, the animals were sacrificed.

RESULTS

Obvious separation of the maxillary incisor teeth was present at the time of sacrifice of the animals but there was no gross difference apparent in the degree of separation between the control and experimental groups. In some instances in all

groups, the rubber wedge had been partially or completely dislodged and these animals were discarded from the study.

In all three series of animals there was found to be considerable variation in the degree of separation of the suture within individual groups. Furthermore, within each series no consistent differences were found in this degree of separation between one group and another. Thus, in one experimental series, the propylthiouracil-treated animals exhibited somewhat greater suture width than thyroxin-treated, while in another series the reverse was true. These findings suggest that the endocrine dysfunction has little influence on the degree of suture separation, the local mechanical stress overshadowing any secondary hormonal effect. It became obvious through further study, however, that cellular activity and bone response at the suture was prominently affected by the state of thyroid function.

In Series I (sixteen animals) the thyroxin-treated animals exhibited greater suture separation than either of the other groups (controls, $155 \pm 51 \mu$; thyroxin, $268 \pm 114 \mu$; thiouracil, $199 \pm 62 \mu$). However, the reaction of the bone on the inner opposing faces of the suture was more dramatic in the propylthiouracil group than in either the control or thyroxin-treated animals (Figs. 1-3). There were no discernible differences in the reaction at the suture between the latter two groups. The reaction in the thiouracil group consisted of a greater thickness of osteoid along the inner suture faces, more prominent osteophyte formation, greater osteoblastic activity and an increased cellularity of the connective tissue between the two plates of bone. No differences were noted in the orientation of the collagen fibres or the vascularity of this connective tissue among the various groups.

In Series II (thirty-one animals) the thyroxin-treated animals demonstrated less suture separation than either the control or the thiouracil-treated groups (controls, $271 \pm 47 \mu$; thyroxin, $222 \pm 42 \mu$; thiouracil, $279 \pm 18 \mu$). However, again the thiouracil-treated animals exhibited greater reaction than either of the two other groups, and the character of the reaction was identical to that seen in Series I.

In Series III (twenty-nine animals) the thiouracil-treated animals exhibited slightly greater separation than either of the other two groups (controls, $377 \pm 104 \mu$; thyroxin, $377 \pm 33 \mu$; thiouracil, $398 \pm 50 \mu$). In this third group, the thiouracil-treated animals similarly demonstrated the most marked suture reaction and this was of the same nature as that described above.

The data in all three series indicate that propylthiouracil evokes a more profound tissue reaction at the site of mechanically-induced suture separation than that seen in either control or hyperthyroid animals.

DISCUSSION

The relationship of thyroxin to skeletal growth previously has been explored mainly with respect to epiphyseal chondrogenesis. The available evidence suggests that thyroxin is primarily concerned with the maturation rather than the proliferation of epiphyseal cartilage (RAY *et al.*, 1950). However, if growth hormone and thyroxin are administered concurrently, a synergistic effect has been noted in hypophysectomized animals. The presence of thyroxin augments the cellular proliferation

that is normally associated with growth-hormone-replacement therapy (MARX, SIMPSON and EVANS, 1942).

Since long bone growth has a chondrogenic component that is not present in sutural growth, it is not surprising that the effects of hypothyroidism and hyperthyroidism on the growth of long bones would not be the same as the effects on sutural growth.

Osteoporosis produced by resorption of both spongy and cortical bone may be associated with hyperthyroidism (BAUER, AUB and ALBRIGHT, 1929). Dried thyroid, moreover, when added to vitamin D deficient diets, increases the development of rickets in experimental animals (MELLANBY, 1923). In this study, however, no increase in resorption rates or failure of bone formation and calcification was noted in the hyperthyroid rats. Similar findings of normal bone development in rapidly growing rats with severe hyperthyroidism have been reported by SMITH and MCLEAN (1938).

A more dramatic osteogenic response was elicited in the artificially expanded suture of hypothyroid rats than in either hyperthyroid or control animals. The degree of activity could not be correlated with the new width of the premaxillary suture following expansion. It is difficult to explain the mechanism for this enhanced cellular activity following the administration of propylthiouracil. It should be stated that apparently more is involved than the toxicity of thyroxine since both normal and hyperthyroid animals showed lower levels of osteogenic response.

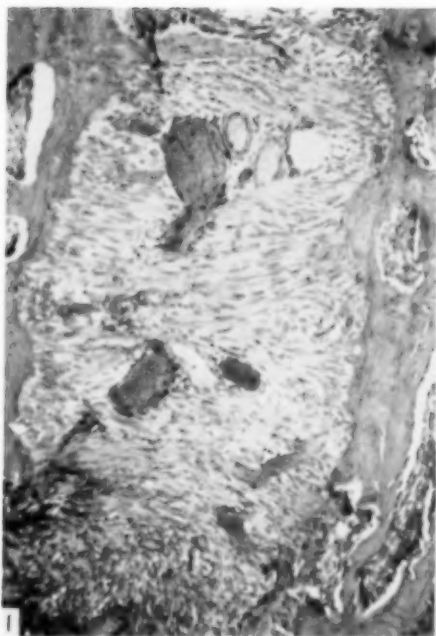
When similar groups of rats with undisturbed premaxillary sutures were studied, no differences were observed among the thyroxine, propylthiouracil and control groups. This suggests that the increased cellular activity in the mechanically expanded suture can more readily demonstrate differences associated with endocrine dysfunction than the less active normally growing suture.

Acknowledgement—This study was supported by a grant (D-353) from the National Institute of Dental Research, United States Public Health Service.

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THE EFFECT OF THYROID FUNCTION ON SUTURAL OSTEOGENESIS IN THE RAT



FIGS. 1 and 2. The range in variation of response during suture separation representative of both control and thyroxin-treated group. Response ranged from almost complete lack of osteophyte formation to numerous new bony spurs and a moderate cellular proliferation.



FIG. 3. Thiouracil-treated animals demonstrated greater bony and cellular reaction to the suture separation.

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AMYLASEAKTIVITÄT UND ATMUNG DES MENSCHLICHEN SPEICHEL

A. KRÖNCKE und C. LÄSSIG

Zahnärztliches Institut der Universität Tübingen, Deutschland

Zusammenfassung—In 97 Speichelproben von 43 Personen wurden die Sauerstoffaufnahme und die Amylaseaktivität bestimmt. Trotz der gemeinsamen Zusammenhänge mit dem Kohlenhydratstoffwechsel ist keine Korrelation der Ergebnisse erkennbar. Dies bedeutet in Übereinstimmung mit früheren Ergebnissen, dass die individuell unterschiedliche Speichelatmung nicht von der Quantität des angebotenen verbrennungsfähigen Substrates bestimmt wird. Die Diskussion dieser Ergebnisse macht ausserdem wahrscheinlich, dass Amylaseaktivität und Zahnkaries nicht miteinander in Beziehung stehen.

Abstract—The oxygen intake and amylase activity of ninety-seven saliva specimens taken from forty-three persons were determined. In spite of the overall correlation with carbohydrate variation, no correlation between the various results is detectable. This, taken in conjunction with previous results, indicates that the individual oxygen intake is not determined by the quantity of the combustible substrate. Discussion of these results, moreover, makes it appear probable that amylase activity and dental caries have no influence on each other.

EINLEITUNG

DIE SPEICHELATMUNG ist im Verlaufe des letzten Jahrzehnts Gegenstand zahlreicher Untersuchungen gewesen (HARTLES und McDONALD, 1950; CALANDRA und ADAMS, 1951; BRAMSTEDT und VONDERLINN, 1952; BURNETT, 1952, 1953, 1954; KRÖNCKE und NAUJOKS, 1954; u.a.). Aus diesen Arbeiten geht unter anderem hervor, dass die Sauerstoffaufnahme des gemischten Nüchternspeichels von fermentativen Umsetzungen abhängt (BRAMSTEDT und VONDERLINN, 1952, 1953), die mit dem Kohlenhydratabbau in nahem Zusammenhang stehen (BRAMSTEDT *et al.*, 1960; KRÖNCKE, 1959a, b). Verantwortlich für diese Stoffwechselprozesse sind offensichtlich die zelligen Elemente, die jedoch in ihren Umsatzleistungen nachhaltig von einem "aktivierenden Prinzip" beeinflusst werden, das im zellfreien menschlichen Speichel enthalten ist (BRAMSTEDT, KRÖNCKE und NAUJOKS, 1957a, b).

Die Grösse der Speichelatmung zeigt individuelle Unterschiede, die mit der Kariesanfälligkeit in Beziehung stehen. Dabei zeichnet sich der Nüchternspeichel eines Kariesresistenten in der Regel durch hohe, derjenige eines Kariesanfälligen durch niedrige Sauerstoffaufnahme aus (KRÖNCKE und NAUJOKS, 1954; KRÖNCKE, 1959b).

Frühere Untersuchungen über den Einfluss abbaufähiger Substrate (Glukose, Fructose, Stärke, Aminosäuren) auf die Speichelatmung wurden von uns so gedeutet, dass die mit der Karies zusammenhängenden Unterschiede der Speichelatmung nicht von der Quantität der vorhandenen verbrennbaren Substrate abzuhängen scheinen

(BRAMSTEDT *et al.*, 1954; KRÖNCKE, 1959a). In diesem Zusammenhang soll nun die Frage beantwortet werden, ob die Aktivität der Speichelamylase durch quantitativ unterschiedliche Substratangebote das Ausmass der Speichelatmung bestimmt. Anlass zu diesen Überlegungen gab nicht zuletzt die Beobachtung von Maltose und Glukose in Nüchternspeichelproben vornehmlich kariesanfälliger Personen (KRÖNCKE, 1959a).

In zweiter Linie soll durch diese Untersuchung festgestellt werden, inwieweit Speichelatmung und Amylaseaktivität in der gleichen Speichelprobe als Kariesanfälligkeitstest identische oder sich widersprechende Ergebnisse aufweisen. Bekanntlich haben FLORESTANO, FABER und JAMES (1941), TURNER und CRANE (1944), SULLIVAN und STORVICK (1950) und in neuerer Zeit nochmals TURNER, ANDERS und BECKER (1957) sowie TURNER (1960) Beziehungen zwischen Amylaseaktivität und Zahnkaries beobachtet. BERGEIM und BARNFIELD (1945) sowie HESS und SMITH (1948) kamen allerdings bei gleichen Untersuchungen zu negativem Ergebnis.

MATERIAL UND METHODEN

I. Die Speichelproben

Gemischter Nüchternspeichel wurde unter weitgehend standardisierten Bedingungen (KRÖNCKE, 1959b) gesammelt. Die Einzelprobe umfasste nicht mehr als 4 ml, sie wurde so bald wie möglich, jedoch nicht später als 3 Stunden nach der Speichelgewinnung dem Experiment zugeführt. Sofern innerhalb dieser Zeit eine Aufbewahrung nötig war, erfolgte diese bei $+4^{\circ}\text{C}$ im Eisschrank.

II. Die Versuchspersonen

Für diese Versuche standen 43 Personen zur Verfügung, die seit über einem Jahr unter ständiger zahnärztlicher Kontrolle stehen. Aufgrund dieser klinischen Überwachung sind zur Zeit 12 Probanden als kariesanfällig (kA) und 23 als kariesresistent (kR) zu bezeichnen, während weitere 8 Personen als fragwürdig kariesanfällig (k?) gelten müssen. Diese klinische Beurteilung ist mit den entsprechenden Werten der Speichelatmung identisch. In Zweifelsfällen wurde die Beurteilung der Kariesanfälligkeit durch diesen Test unterstützt.—Von den 43 Versuchspersonen wurden insgesamt 97 Speichelproben zur Auswertung herangezogen.

III. Die Speichelatmung

Die Speichelatmung wurde im Warburg-Apparat bei $37,0^{\circ}\text{C}$ und einer Schüttelfrequenz von 120/min bestimmt. Diese spezielle Methodik darf als bekannt vorausgesetzt werden, sie ist u.a. von UMBREIT, BURRIS und STAUFFER (1949) ausführlich beschrieben und erläutert worden.—Der Ansatz enthielt jeweils 1 ml Speichel und 2 ml Veronalpuffer (pH 7,0).

IV. Die Amylaseaktivität

Die Amylaseaktivität wurde mit Hilfe der Jod-Stärke-Reaktion kolorimetrisch gemessen. Hierfür wurde ein für Serumuntersuchungen ausgearbeitetes Verfahren von SMITH und ROE (1949) wie folgt modifiziert:

Reagentien. Stärkelösung 0,5% (Amyl.solani, Merck), Phosphatpuffer (pH 7,2) (72,6 ml m/15 sek. Natriumphosphat + 27,4 ml m/15 prim. Kaliumphosphat), Natriumchlorid 3 %, n/l Salzsäure, n/10 Jodlösung.

Ausführung. 5 ml Stärkelösung, 3 ml Phosphatpuffer und 1 ml Kochsalzlösung werden gut durchmischt und 15 Min bei 37°C vortemperierte. 1 ml zentrifugierter und auf 1:500 verdünnter Speichel wird zugesetzt; der zu jeder Speichelprobe gehörige Kontrollansatz erhält zu gleicher Zeit 2 ml Salzsäure. Alle Ansätze werden sorgfältig durchmischt und 30 Min bei 37°C inkubiert. Sofort hieran anschliessend werden auch die Speichelansätze durch Zugabe von 2 ml Salzsäure inaktiviert.

Jedem Ansatz werden 0,8 ml entnommen und in bereits vorbereitete Messkolben überführt, die 40 ml H₂O, 0,5 ml n-HCl und 0,1 ml Jodlösung enthalten und anschliessend auf 50 ml aufgefüllt werden. Extinktionsmessung der blau gefärbten Lösungen im Photometer "Eppendorf" (Schichtdicke 20 mm, Filter Hg 578 nm, Messung gegen H₂O).

Die Berechnung erfolgt direkt aus den Extinktionen des Speichelansatzes (ES) und des Kontrollansatzes (EK); Multiplikation des Ergebnisses entsprechend der Speichelverdünnung mit 5000:

$$\frac{EK - ES}{EK} \cdot 5000 = \text{Amylaseeinheiten/ml Speichel.}$$

Wegen der hohen Aktivität der Speichelamylase werden alle Ergebnisse in der vorliegenden Arbeit in 10³ Amylaseeinheiten angegeben.

V. Statistik

Alle Messergebnisse wurden nach den für Stichproben mit unterschiedlicher Anzahl der Einzelwerte gebräuchlichen Methoden statistisch behandelt (WEBER, 1956). Hierbei ist N=Anzahl der Einzeluntersuchungen, \bar{x} =arithmetischer Mittelwert, s_x =Standardabweichung des Mittelwertes, t =Ergebnis des t -Testes, P =Irrtumswahrscheinlichkeit.

ERGEBNISSE

Bei 97 Nüchternspeichelproben von kariesresistenten (kR), kariesanfälligen (kA) und fragwürdig kariesanfälligen (k?) Personen wurden die Sauerstoffaufnahme (Speichelatmung) und die Amylaseaktivität bestimmt und miteinander verglichen. Nach Kariesanfälligkeit und Kariesresistenz geordnet unterscheiden sich dabei die Mittelwerte wie auch die Einzelwerte der Speichelatmung mit hoher Signifikanz (siehe Tab. 1). Die Mittelwerte der Amylaseaktivität liegen im Gegensatz hierzu dicht beieinander, die Unterschiede sind nicht signifikant. Die in jeder Gruppe angegebenen grössten und kleinsten Einzelwerte zeigen zudem, dass die Amylaseaktivität in allen drei Gruppen unabhängig vom Grade der Kariesanfälligkeit über einen annähernd gleichen Bereich streut (siehe Tab. 1).

Die Schwankungen der Amylaseaktivität sind bei Speichelproben einer Person, die an verschiedenen Tagen gewonnen wurden, recht erheblich. Sie betrugen bei unseren Untersuchungen zwischen 50 und 400 % (vom niedrigsten Messwert aus

TABELLE 1. VERGLEICH DER SAUERSTOFFAUFNAHME UND DER AMYLASEAKTIVITÄT DES NÜCHTERNSPEICHELKARIESRESISTENTER (kR), KARIESANFÄLLIGER (kA) UND FRAGWÜRDIG KARIESANFÄLLIGER (k?) PERSONEN

		kR	k?	kA
Anzahl der Bestimmungen N		37	14	46
Speichelatmung ($\mu\text{l O}_2/\text{Std.}$)	x_{\max}	275	90	65
	x_{\min}	91	66	5
	\bar{x}	149,3	78	41,9
	$s_{\bar{x}}$	$\pm 9,1$	$\pm 2,1$	$\pm 1,8$
	t $P (\%)$	4,77 <0,1	11,34 <0,1	11,7 <0,1
Amylaseaktivität (10^3 Einh.)	x_{\max}	4,48	4,45	4,86
	x_{\min}	0,09	0,01	0,04
	\bar{x}	2,39	2,15	2,27
	$s_{\bar{x}}$	$\pm 0,23$	$\pm 0,40$	$\pm 0,27$
	t $P (\%)$	0,54 >5	0,36 >5	0,25 >5

TABELLE 2. EINIGE BESTIMMUNGEN DER SPEICHELATMUNG UND DER AMYLASEAKTIVITÄT IN GLEICHEN NÜCHTERNSPEICHELPROBEN AN VERSCHIEDENEN TAGEN
(Die Abweichung wurde in % jeweils vom niedrigsten zum höchsten Wert berechnet)

	Pers.	Speichelatmung ($\mu\text{l O}_2/\text{Std.}$)				Amylaseaktivität (10^{-3} Einh.)			
		1	2	3	%	1	2	3	%
kR	U.M.	142	132	—	8	2,46	1,67	—	47
	H.M.	235	235	275	17	2,51	4,57	4,48	82
	U.K.	153	125	133	15	1,23	1,86	1,05	77
	D.P.	220	216	—	2	1,27	3,85	—	203
	LS.	196	228	—	11	0,70	3,57	—	410
kA	A.K.	42	64	59	41	1,64	1,73	3,89	137
	G.E.	62	55	48	29	0,45	0,80	1,89	320
	H.L.	65	51	42	55	2,32	3,84	3,88	67
	J.H.	49	32	—	53	0,66	0,33	—	100
	H.P.	39	41	31	26	0,27	1,18	0,59	337

berechnet). Die Tabelle 2 zeigt dies an einigen Beispielen. Im Gegensatz zur Amylaseaktivität waren die Schwankungen der Speichelatmung der gleichen Speichelproben zwischen 2 und 55 % so gering, dass sie in keinem der dargestellten Fälle die Stichhaltigkeit des Kariesanfälligkeitstestes infrage stellen würden.

Beim Vergleich der zu jeweils einer Person gehörigen Messwerte untereinander lässt sich keine Gesetzmässigkeit zwischen den Ergebnissen der Speichelatmung und denen der Amylaseaktivität feststellen.

DISKUSSION

Die Ergebnisse zeigen eindeutig, dass Atmung und Amylaseaktivität weder in gleichen Speichelproben noch im Durchschnitt repräsentativer Stichprobenkollektive in irgendeiner erkennbaren Beziehung zueinander stehen. Wirksamkeit und Aktivität der Speichelamylase bestimmen demnach nicht, wie man annehmen könnte, den Glukosedurchsatz im Speichel, der übereinstimmend mit der unterschiedlichen "oral sugar clearance" (LUNDQVIST, 1952; SWENANDER-LANKE, 1959) im Speichel kariesanfälliger Personen langsamer abläuft als bei Kariesresistenten (BRAMSTEDT *et al.*, 1960). Aus diesem Befund geht auch übereinstimmend mit früheren Ergebnissen hervor, dass die Speichelatmung in ihrem individuell unterschiedlichen Ausmass nicht oder jedenfalls nicht allein vom Angebot verbrennbarer Substrate bestimmt wird (BRAMSTEDT *et al.*, 1957a, b).

Betrachtet man Speichelatmung und Amylaseaktivität in ihrer Eignung als Kariesanfälligkeitsteste, so stimmen die Ergebnisse beider Teste sowohl bei den Einzelwerten als auch bei den Gruppendurchschnitten nicht überein. Bekanntlich haben auch BERGEIM und BARNFIELD (1945) sowie HESS und SMITH (1948) bei klinischen Überprüfungen, neuerdings auch ROSEN *et al.* (1957) durch Tierexperimente keinen Zusammenhang zwischen Kariesanfälligkeit und Amylaseaktivität erkennen können. Im gleichen Sinne sind die Beobachtungen von BATES (1958) verständlich, der bei 6 untersuchten Personen nach vierwöchentlicher Reduktion des Kohlenhydratkonsums keine Änderung der Amylaseaktivität im Parotis-Speichel feststellen konnte.

Auch gegen die Stichhaltigkeit der Speichelatmung als Kariesanfälligkeitstest sind kürzlich von GREEN, KAY und CALANDRA (1959) Einwände erhoben worden, die indes wegen erheblicher experimenteller Abweichungen (Verwendung von Phosphatpuffer, Verdünnungseffekt der Speichelprobe) keine Vergleiche mit unseren Untersuchungen erlauben (KRÖNCKE, 1959b). Stattdessen ist hervorzuheben, dass nach neueren Untersuchungen Speichelatmung und Pufferkapazität des Speichels in hohem Masse miteinander korrelieren (KRÖNCKE, 1961). Dies ist insofern von besonderer Bedeutung, als die Pufferkapazität des Speichels derzeit als verlässlichster Kariesaktivitätstest gilt (ERICSSON, 1959). Die vorliegenden Befunde machen es deshalb sehr wahrscheinlich, dass zwischen der Aktivität der im menschlichen Speichel in der Regel sehr hochwirksamen Amylase und der Zahnkaries kein Zusammenhang besteht.

Dies steht anscheinend in Gegensatz zu den positiven Befunden, die TURNER (1960) nach sehr gründlichen und über drei Jahre laufenden Versuchen kürzlich veröffentlichte. Bei unseren Untersuchungen wollten wir jedoch zunächst prüfen,

inwieweit Speichelatmung und Amylaseaktivität im Nüchternspeichel kariesresistenter oder kariesanfälliger Personen miteinander korrelieren. TURNER's Bericht zeigt, dass es sehr wertvoll sein dürfte, in weiteren experimentellen Studien auch Vergleiche mit der Amylaseaktivität in Speichelproben anzustellen, die nach seiner Methodik vormittags (10 Uhr) gesammelt werden.

Anerkennung—Diese Untersuchungen wurden durch Sachbeihilfen der Deutschen Forschungsgemeinschaft, Bad Godesberg, und der Research Corporation, New York, in dankenswerter Weise unterstützt.

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CITRATE IN MINERALIZED TISSUES—III

THE EFFECT OF PURIFIED DIETS LOW IN CALCIUM AND VITAMIN D ON THE CITRATE CONTENT OF THE RAT FEMUR

R. L. HARTLES and A. G. LEAVER*

Biochemistry Department, School of Dental Surgery,
University of Liverpool

Abstract—A diet low in Ca (<0.03 per cent) but adequate in vitamin D when fed to the rat results in a 50 per cent elevation of bone citrate above the normal value. A diet deficient in vitamin D but adequate in calcium causes a small drop in bone citrate. A diet deficient in both vitamin D and calcium results in a bone citrate value which is only half the normal figure. Calcium and vitamin D mutually mitigate a deficiency of the other in maintaining the synthesis of bone citrate.

THE CITRATE CONTENT of bone is affected by the calcium nutrition of the animal and by the presence or absence of vitamin D, but the nature of this relationship is not very clear. STEENBOCK and BELLIN (1953) conclude that the amount of citric acid in the rat femur is a function of the state of calcium nutrition of the animal rather than of the vitamin D content of the diet. CARLSSON and HOLLUNGER (1954) found on the other hand that in rats given a diet very low in calcium (0.04 per cent) the citric acid concentration in the femur increases by about 70 per cent in 4 days after the administration of vitamin D even though the calcium intake remains low. They also found that in the continued absence of vitamin D an increase in dietary calcium results in an increased bone citrate.

In the present experiment rats were maintained on four purified diets, a control diet adequate in the known nutritional factors, a diet deficient in calcium, a diet deficient in vitamin D, and a diet deficient in both calcium and vitamin D. The findings were that in the presence of adequate calcium a lack of vitamin D had little effect on the amount of citrate in the rat femur. A deficiency of calcium and vitamin D reduced the citrate content of the femur to half the control value. In the presence of adequate vitamin D but with a deficiency of calcium the bone citrate was raised 50 per cent above the control level.

TABLE I. THE MAIN FEATURES OF THE FOUR DIETS

Group	Diet	Calcium	Phosphorus	Vitamin D
A	HS 7	Normal	Normal	Normal
B	HS 8	Low	Normal	Normal
C	HS 9	Normal	Normal	Low
D	HS 10	Low	Normal	Low

* Nuffield Dental Research Fellow.

EXPERIMENTAL

Animals. Forty month-old animals (20 male and 20 female) were distributed between four equal groups.

Diets. Four diets were prepared as shown in Table 1.

HS7—control diet, contained 0.56 per cent Ca, each 960 g of diet contained

Powdered sucrose	670 g
Albumin	200 g
Groundnut oil	50 g
Salt mixture A	40 g

To each 960 g of diet were added

Thiamine hydrochloride	15 mg
Riboflavin	15 mg
Pyridoxine	5 mg
Nicotinamide	25 mg
<i>p</i> -Aminobenzoic acid	500 mg
Choline chloride	1500 mg
Vitamin A acetate	20 mg
α -Tocopheryl acetate	250 mg
Menaphthone	20 mg
Calciferol	200 μ g

HS8—low calcium, normal vitamin D. This diet was compounded as HS7 with the substitution of salt mixture B for salt mixture A. The diet contained <0.03 per cent Ca.

HS9—normal calcium low vitamin D. This diet was prepared as HS7 but with the omission of calciferol; it contained 0.56 per cent Ca.

HS10—low calcium, low vitamin D. This diet was prepared as HS8 but with the omission of calciferol; it contained <0.03 per cent Ca.

Salt mixtures

	A	B
CaCO ₃	300	—
K ₂ HPO ₄	470	402
KH ₂ PO ₄	—	290
NaH ₂ PO ₄ .2H ₂ O	—	275
NaCl	670	575
CaHPO ₄	680	—
KCl	115	100
MgSO ₄	100	85
Ferric citrate	55	48
KI	1.6	1.5
MnSO ₄ .4H ₂ O	9	8
ZnCl ₂	4	3.2
CuSO ₄ .5H ₂ O	2.4	2.0
CoCl ₂	0.2	0.2

Salt mixture A contained 13.3 per cent calcium and 9.9 per cent phosphorus; mixture B contained no added calcium salts and 10.8 per cent phosphorus. The proportions of salts were adjusted so that the ratio of sodium to potassium was roughly the same in each mixture.

The diets and de-ionized water were given to the animals in unrestricted amounts.

As shown in Table 1, group A received diet HS7, group B HS8, group C HS9 and group D HS10.

Housing. Five rats were kept in each wire mesh cage with $\frac{1}{8}$ in. mesh floor, and were weighed twice weekly.

Duration of experiment. The animals were maintained on their respective diets for 8 weeks. By this time four animals in group D were showing signs of tetany; all animals were therefore killed by ether inhalation.

Preparation of bones for analysis. The femora were dissected as cleanly as possible. They were broken into fragments and extracted with ethanol and ether for 6 hr in a Soxhlet continuous extraction apparatus.

The fragments from each pair of femora were pooled, dried at 110 C for 3 hr and ground to a powder in a percussion mortar. The resulting powder was then separated from soft tissue and bone marrow by flotation in an acetone/bromoform mixture of specific gravity 1.5 (HARTLES, 1951). All the analytical data refer therefore to dry fat free material with a specific gravity not less than 1.5.

Analytical methods. The material obtained from each pair of femora was analysed for calcium, phosphorus, nitrogen and citrate. Analyses for calcium and phosphorus were carried out as described by HARTLES (1951), nitrogen and citrate as described by HARTLES and LEAVER (1960). (See also LEAVER, EASTOE and HARTLES, 1960).

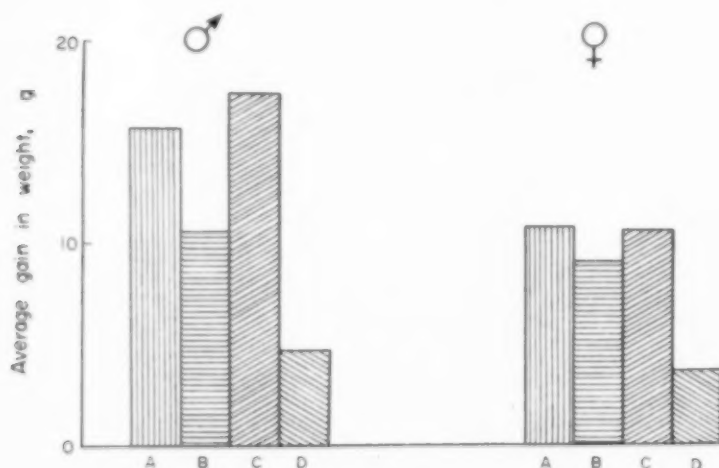


FIG. 1. Average gain in weight per rat per week. A, Control; B, Deficient in calcium; C, Deficient in vitamin D; D, Deficient in calcium and vitamin D.

RESULTS

Growth of animals. The average weekly gains in weight over the 8-week experimental period are given in Fig. 1. It is noticeable that there was little difference in the weights of animals in groups A and C, showing that deprivation of vitamin D in the presence of adequate calcium and a Ca/P ratio of 1.34 (w/w) permitted the rat to grow normally. Group B animals who received the diet deficient in calcium grew less well than the control group A, but very much better than the animals of group D who were deficient in both calcium and vitamin D.

Signs of tetany. After exactly 8 weeks on the diet four animals in group D showed severe signs of tetany (Fig. 2), the onset of which was sudden. The hind quarters of the affected animals were extended and rigid. When placed on a flat surface vigorous movements of the fore legs caused the animals to spin round dragging their immobile hind quarters. At this time the remaining animals in the group were hyperexcitable and so the whole experiment was terminated.

Analytical data. The results are presented in Table 2. The animals of groups B and D were seriously affected. The percentage of calcium and phosphorus in the bones was very much lower than in the control animals and the nitrogen values were raised. The mineralization of the bones of the animals in group C was slightly less than the controls and the nitrogen content slightly more. These differences although small are highly significant ($P < 0.001$).

TABLE 2. PERCENTAGE COMPOSITION OF DRY FAT FREE RAT FEMUR OF A SPECIFIC GRAVITY NOT LESS THAN 1.5.

Each value is the mean of the results from ten animals \pm standard error of the mean

Group	Ca	P	N	Citric acid
A—control	24.30 \pm 0.19	12.11 \pm 0.11	3.78 \pm 0.02	0.506 \pm 0.005
B—low calcium	19.69 \pm 0.19	9.93 \pm 0.15	5.88 \pm 0.06	0.756 \pm 0.016
C—low vitamin D	23.53 \pm 0.04	11.59 \pm 0.06	3.93 \pm 0.03	0.457 \pm 0.010
D—low calcium low vitamin D	19.92 \pm 0.18	9.74 \pm 0.09	5.94 \pm 0.05	0.244 \pm 0.014

There was no significant difference in the percentages of calcium, phosphorus and nitrogen between groups B and D.

Citric acid. The femora of group C animals contained about 10 per cent less citrate than the controls. This difference though not large is highly significant ($P < 0.001$). The bone samples of group D animals contained only half as much citrate as the controls whereas in group B animals the bone contained 50 per cent more citrate than the controls and three times as much as group D animals.

DISCUSSION

Perhaps the most interesting finding in this experiment concerned the animals maintained on diets low in calcium (groups B and D). The animals of group D did not receive vitamin D and after 8 weeks on the diet were exhibiting signs of tetany.

Their bone citrate was low, 0.244 per cent; this corresponds to 12.3 mg citrate per g of calcium. The animals of group B received vitamin D (200 μ g calciferol to each 960 g diet) and their bone citrate was raised threefold to 0.756 per cent or 38.4 mg of citrate per g of calcium. There was no significant differences in the calcium, phosphorus or nitrogen content of the bones of these two groups. Taken in isolation this piece of evidence suggests a direct effect of vitamin D on the citrate content of bone. Turning to study the results for groups C and D, both of which received diets deficient in added vitamin D, it will be noticed that the citrate in the bones of the animals of group C (normal calcium) was almost double that in group D. Thus the addition of calcium to diets low in vitamin D caused an increase in the deposition of bone citrate, together with an increase in calcium and phosphorus and a drop in nitrogen percentages. Here there seems to be a direct effect of calcium on bone citrate.

However if the data for groups A and B are examined it becomes apparent that the relationship is not so simple. Groups A and B both had adequate vitamin D, the deprivation of calcium (group B) resulted in an increased bone citrate when compared with that of the control animals (group A).

Deprivation of vitamin D, in the presence of adequate calcium as in group C, resulted in a small but highly significant drop in calcium, phosphorus and citrate and a significant rise in nitrogen.

The highest level of bone citrate occurred in those animals which received a low calcium diet with adequate vitamin D. This is a puzzling observation. CARLSSON and HOLLUNGER (1954) maintained rats on diets low in calcium for periods of 3 weeks. They reported that in two separate groups deprived of vitamin D, in addition to low calcium intake, the citrate content of the tibia-fibula was in one group 20.7 mg/g Ca and in the other 13.9 mg/g Ca. Animals receiving a similar diet but with the addition of vitamin D had a bone citrate of 44.5 mg/g Ca. These figures are comparable with our 12.3 and 38.4 mg/g Ca without and with the vitamin. These workers did not comment on this very high citrate level. The present work also confirms their finding that in the absence of vitamin D the bone citrate is doubled when the diet contains adequate calcium. Both calcium nutrition and vitamin D influence the deposition of citrate in bone, and as stated by NICOLAYSEN and EEG-LARSON (1956) it appears that a defect arising from a deficiency of vitamin D is mitigated to a large extent when the diet is rich in calcium and phosphorus.

NEUMAN and NEUMAN (1958) have put forward a tentative hypothesis in which they postulate a relationship between vitamin D, parathyroid hormone and citrate. They suggest that vitamin D promotes the synthesis of citrate from pyruvate and oxalacetate, possibly by activating or synthesizing one of the necessary co-factors, e.g. diphosphopyridine nucleotide. They also postulate that parathyroid hormone inhibits triphosphopyridine nucleotide-linked reactions which would lead to inhibition both of the pentose-oxidation shunt reactions and of the further metabolism of citrate via iso-citrate. DIXON and PERKINS (1952) demonstrated that the enzymes necessary for the production of citrate were present in bone and cartilage but that iso-citric dehydrogenase activity was not measurable in bone and low in cartilage. Thus the

enzymes necessary for the further metabolism of citrate are either lacking or present in very low concentration in bone. These results are from normal animals and there does not appear to be any data available concerning the enzymes of bone formed under physiological stress. If iso-citric dehydrogenase activity is indeed not measurable in bone it is difficult to imagine parathyroid hormone having any great influence on this reaction.

It is of interest to examine the present results in the light of the Neuman's hypothesis, even though such examination can be little more than speculation. The control animals (group A) may be deemed to be normal with a bone citrate of about 0.5 per cent. Group B animals received adequate vitamin D but very little calcium. It is reasonable to assume that in order to maintain the circulating calcium the parathyroids would be stimulated to an activity greater than normal, and according to the Neuman's hypothesis since vitamin D is present the production of citrate should be increased and accumulate in the bone. This was so; the bones of group B animals had an average citrate content of 0.75 per cent, a rise of 50 per cent compared with the control animals.

The data for groups C and D are less easily reconciled with the hypothesis. Both groups received diets very low in vitamin D, so that in neither case should there be a stimulation of citrate production. The parathyroids of the animals of group D (low calcium) should be stimulated more than those of group C. If the Neuman's hypothesis has been interpreted correctly the citrate content in the bones of group D animals might be expected to have more citrate than those of group C. This was not found. The results are therefore not in complete accord with the Neuman's hypothesis, but confirm the results of CARLSSON and HOLLUNGER (1954) that bone citrate is raised when vitamin D is added to the diet of calcium deficient rats and when calcium is added to the diets of vitamin D deficient animals. The results also support the view of NICOLAYSEN and EEG-LARSON (1956) that calcium and vitamin D are each able to counteract a deficiency of the other in maintaining bone citrate.

In undermineralized bone (groups B and D) citrate was low only in the absence of vitamin D. Where mineralization was only slightly impaired (group C) the bone citrate was only slightly although significantly lowered. The relationship between vitamin D and bone citrate is a complex one. A study of the effect of hypervitaminosis D in the presence and absence of calcium on the bone citrate may help to clarify this relationship.

Acknowledgements—The authors wish to thank Mr. R. P. WILLIAMS for his care and maintenance of the animals, and Mr. J. S. BAILIE for the photograph.

Grateful thanks are due to Messrs. J. BIBBY and Sons Ltd. for the gift of groundnut oil and to Messrs. TATE and LYLE Ltd. for the gift of icing sugar.

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FIG. 2. A rat showing signs of tetany. The animal had received a diet deficient in calcium and vitamin D for 8 weeks.

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FACTORS GOVERNING WATER BINDING BY HYDROXYLAPATITE

H. M. MYERS

School of Dentistry, University of California, San Francisco Medical
Center, California, U.S.A.

Abstract—Water vapour adsorption by hydroxylapatite crystals is influenced by the presence or absence of small interstitial crevices capable of retaining water by capillarity. At low pressures where adsorption is more nearly monomolecular, the presence of such crevices is of no consequence. Alterations in the surface composition of apatite brought about by heterionic exchange brings about different amounts of water adsorption.

INTRODUCTION

A NUMBER of surplus ions foreign to the hydroxylapatite lattice are associated with biological apatites. It has been suggested that many of these foreign ions are located in the "hydration shell" of the crystallites rather than in the lattice proper (SOBEL, 1960). Attention has therefore been directed to the nature, composition and mechanism of binding of the "hydration shell" and the crystal surface. The investigations reported in this paper deal with some of these questions.

That apatite crystals retain water against strong centrifugal forces tending to remove it has been reported by MYERS (1957a). Such studies as well as others which have demonstrated the adsorption of water vapour by packed apatite crystals (NEUMAN, TORIBARA and MULRYAN, 1953) do not distinguish between retention of water on the crystal surface *per se* and condensation in minute interstices between adjacent crystals. The role of capillarity in adsorption phenomena in general has not been entirely clarified. It is generally accepted that vapours under low pressure may adsorb on surfaces as monolayers (DE BOER, 1953). So-called multimolecular adsorption is more controversial inasmuch as higher pressures are required and at such pressures capillarity becomes progressively more important (BRUNAUER, 1945). The apparent binding of water as vapour or liquid by apatite crystallites may therefore not be a clear demonstration of the existence of a hydration shell since it might well be due to capillarity. All measurements of adsorption by hydroxylapatite crystallites must of necessity utilize a mass of packed crystals with a presumed large but unknown interstitial void between them. It is of interest to inquire whether variations in the size of the void between crystallites will change the amount of adsorbed water. Increasing the void space of a given mass of crystals should reduce the number of small crevices capable of filling by capillarity and thereby reduce the total adsorption. At vapour pressures low enough to allow monolayers to form, variations in the size

of interstitial crevices should have a negligible effect. Under conditions of higher pressure, which favour capillary condensation, the amount of adsorption should be appreciably reduced.

MATERIALS AND METHODS

100 mg of commercial synthetic hydroxylapatite (Victor Chemical Works, 155 N. Wacker Dr., Chicago 6, Ill., Ca/P 1.66, density 3.14) were dried overnight at 110°C. The sample was then dispersed in a weighed dry Petri dish cover over areas of 1, 9 and 25 cm². The areas were denoted by an inked grid of 1 cm squares. The dishes were then placed in the upper compartments of air-tight dessicators in which water or an NaOH solution in the lower compartment had been allowed to equilibrate at 25°C. Where the equilibrating solution was distilled water, the vapour pressure of H₂O in the upper chamber was 23.76 mm of Hg and this was taken as 100 per cent relative humidity. Where the equilibrating solution was 24.66% NaOH the vapour pressure of the water was reduced to 60 per cent of the distilled H₂O value. Intermediate values of vapour pressure were obtained by controlling the concentration of the NaOH solution in the lower compartment of the dessicator according to standard tables (LANGE, 1956). The Petri dishes were weighed every 2 days until a constant weight was reached. This required from 3 to 4 weeks. Fluctuations in weight which occurred while using the balance were compensated for by averaging the final three weights. Successive determinations are reproducible within 14 per cent of the mean.

TABLE I

Relative humidity (%)	Vapour pressure (mm Hg at 25°C)	Water (mM per g apatite)
100	23.76	22.0
90	21.35	4.0
80	18.97	2.4
70	16.60	1.4
60	14.23	1.0

RESULTS

Table I indicates the adsorption of water vapour at 60–100 per cent r.h. for apatite crystals occupying an area of 1 cm². The lowest adsorption, at 60 per cent r.h., is 1 mm for each gram of solid. Using the conventional value of 10.5 Å² for the area of a water molecule (ADAM, 1941), the area occupied by 1 mm of water as a monolayer is 63.0 M². 1 g of the apatite sample used has an area of 65.9 M² as determined by the BET method (MYERS, 1957b). The striking correspondence between the two areas indicates that the existence of a monolayer of water on apatite at 60 per cent r.h. is consistent with the data. As the vapour pressure of water was increased the adsorption increased slowly. It rose abruptly at 100 per cent, suggesting the occurrence of capillary condensation at saturation pressures.

The effect of differing degrees of dispersion of crystals on adsorption is shown in Table 2. It is clear that as a given mass of crystals is more widely scattered, its adsorption at 100 per cent r.h. is appreciably reduced. No such pattern is seen for adsorption at 60 per cent r.h.

TABLE 2

Area occupied by 100 mg apatite (cm ²)	mM H ₂ O ads. by 1 g apatite	
	100 % r.h.	60 % r.h.
1	21.3	0.9
9	18.1	0.9
25	13.6	1.0

The data suggest that at least 36 per cent of the adsorption of water by packed apatite crystallites is due to capillarity. Since increasing dispersion even more may reduce adsorption to a greater extent, this figure must be regarded as a minimum.

If the implications of the above data are correct and a monolayer of water exists on the surface of apatite at 60 per cent r.h., it might be expected that some specific relationship between the composition of the crystal surface and water binding exists. Heterionic exchange may therefore alter this relationship and the amount of water adsorbed. To test this hypothesis, 100 mg apatite samples were first equilibrated at 25°C for 48 hr with 100 ml of a pH 7.0, ionic strength 0.16 solution containing a variety of cations capable of exchanging with surface ions of the apatite lattice. The anion was always chloride unless otherwise indicated. The apatite was recovered with millipore filters, dried overnight at 100°C and redispersed as a powder by lightly grinding in a mortar and pestle. It was then exposed to 60 per cent r.h. as before. Table 3 presents the results of these findings. All values are the averages of four separate determinations.

TABLE 3

Treatment of apatite	mM H ₂ O per gram at 60% r.h.
No equilibration	1.0
Equilibration with BaCl ₂	1.0
SrCl ₂	1.3
MgCl ₂	1.4
NaCl	1.5
NaF	0.8
KCl	1.0
KF	0.6

DISCUSSION

As a first approximation, it appears that presumed substitution of a cation for Ca^{++} increases adsorption while substitution of an anion for hydroxyl lowers it. It is doubtful that Ba^{++} or K^+ are able to exchange with surface Ca^{++} while Mg^{++} , Sr^{++} and Na^+ can (NEUMAN and NEUMAN, 1958). Likewise F^- is believed to exchange with OH^- groups and this is supported by evidence that the pH of the equilibrating solution containing fluoride increased about 1 pH unit after exposure to apatite.

It is concluded that the surface composition of apatite crystals does have an effect on the quantity of water adsorbed. Such water may well represent a portion of the hydration shell. Earlier estimates of the size of the hydration shell were probably too high since they included water retained by capillarity.

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EFFECT OF ADMINISTRATION OF EDTA BY VARIOUS ROUTES ON DENTAL CARIES IN THE RAT

POSSIBLE ROLE OF COPROPHAGY

RACHEL H. LARSON*, I. ZIPKIN and M. RUBIN†

National Institute of Dental Research, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland, U.S.A.

Abstract—A significant increase in experimental rat caries has been demonstrated due to the administration of EDTA in the diet and by intubation, a slight but not significant increase when given by injection. It is pointed out that the effect of coprophagy and different eating habits must be considered in the interpretation of the results.

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1961

THE INCORPORATION of ethylene diamine tetra-acetic acid (EDTA) in various test diets has been associated with an increase in the severity of dental caries on both occlusal (ZIPKIN, 1953; ZIPKIN and LARSON, 1959) and smooth surfaces (STEPHAN and HARRIS, 1953; LARSON, 1959; ZIPKIN and LARSON, 1959). The mechanism of this action is as yet incompletely understood. Other associated effects of EDTA are a reduction in food intake, impaired weight gain and interference with iron utilization indicated by a reduction in haemoglobin and a decrease in incisor pigmentation. These findings raise the question as to whether the increase in caries activity is due to a local action of EDTA in the oral cavity, to adverse systemic effects or to a combination of both. In an attempt to resolve these possibilities, we have studied the effects of EDTA administered in the diet, by stomach tube and by intraperitoneal injection. These results are discussed primarily with regard to the problem of coprophagy in experimental caries studies.

METHODS

The study consisted of two experiments. Weanling litter-mated Osborne-Mendel rats from the N.I.H. colony were individually housed in screened-bottom cages, under the experimental variations indicated in Tables 1 and 2. Diets containing 0.33 per cent sodium EDTA in Experiment A and 0.36 per cent in Experiment B were administered for 5 consecutive weekdays. Aqueous solutions of 3.3% and 3.6% sodium EDTA for Experiments A and B respectively were used for both intubation and injection to provide proportional amounts of sodium EDTA by extra-oral routes based on daily intake of diet.

* Taken in part from a thesis submitted in partial fulfilment of the requirements for the Ph.D. degree in Chemistry at Georgetown University, Washington, D.C., June 1958.

† Georgetown University, Washington, D.C.

TABLE 1. EXPERIMENT A. CARIES RESULTS, DIET INTAKES, WEIGHTS GAIN, INCISOR PIGMENTATION AND TERMINAL HAEMOGLOBIN OF RATS TRIO-FED DIET 580 (Average per rat)

	Pair fed control	EDTA in diet	EDTA by intubation
No. of animals	18	18	18
Percentage with caries	100	100	100
No. of carious teeth	$7.6 \pm 0.56^*$	10.0 ± 0.58	9.6 ± 0.43
No. of carious areas:			
bucco-lingual, enamel	0.0	9.1 ± 2.3	6.6 ± 2.3
bucco-lingual, dentine	0.0	3.6 ± 1.6	0.7 ± 0.51
approximal, enamel	1.5 ± 0.57	8.5 ± 1.5	6.9 ± 0.93
approximal, dentine	0.0	2.3 ± 0.80	1.1 ± 0.53
sulcal, enamel	14.7 ± 1.9	24.1 ± 2.7	20.9 ± 2.3
sulcal, dentine	1.7 ± 1.0	10.3 ± 1.9	8.8 ± 1.9
Daily weight gain (g)	1.3 ± 0.04	1.3 ± 0.05	1.3 ± 0.05
Daily diet intake (g)	8.0 ± 0.09	8.0 ± 0.10	8.0 ± 0.11
Daily EDTA intake (mg)	0.0	13.6	13.6
Incisor pigmentation score	3.6 ± 0.18	0.0	0.05 ± 0.06
Terminal haemoglobin (g %)	16.1 ± 0.21	7.6 ± 0.41	10.1 ± 0.47

* Standard error.

TABLE 2. EXPERIMENT B. CARIES RESULTS, DIET INTAKES, WEIGHT GAIN AND INCISOR PIGMENTATION OF RATS FED DIET 633 (*ad libitum*) (Average per rat)

	Control	EDTA in diet	EDTA by intubation	EDTA by injection
No. of animals	34	33	28	31
Percentage with caries	100	100	100	100
No. of carious teeth	$6.8 \pm 0.46^*$	9.2 ± 0.41	8.5 ± 0.50	7.2 ± 0.59
No. of carious areas:				
bucco-lingual, enamel	9.2 ± 1.2	21.1 ± 0.72	21.3 ± 3.1	14.5 ± 2.7
bucco-lingual, dentine	2.2 ± 0.50	6.3 ± 1.8	6.4 ± 1.4	3.6 ± 1.1
approximal, enamel	2.4 ± 0.72	5.4 ± 0.95	5.6 ± 1.1	3.1 ± 0.85
approximal, dentine	0.0	0.0	0.5 ± 0.26	0.06 ± 0.06
sulcal, enamel	6.0 ± 0.96	11.3 ± 1.2	12.9 ± 2.3	8.2 ± 1.3
sulcal, dentine	0.06 ± 0.06	1.5 ± 0.40	2.7 ± 1.3	0.7 ± 0.30
Daily weight gain (g)	3.7 ± 0.06	3.0 ± 0.10	3.4 ± 0.10	2.4 ± 0.08
Daily diet intake (g)	14.3 ± 0.21	12.0 ± 0.27	13.3 ± 0.26	10.0 ± 0.21
Daily EDTA intake (mg)	0.0	22.6	25.3	19.0
Incisor pigmentation score	3.1 ± 0.08	0.1 ± 0.07	2.2 ± 0.20	2.9 ± 0.40

* Standard error.

Experiment A

Twenty litters of female rats were trio-fed Diet 580 (32 per cent skim milk powder, 66 per cent sucrose and 2 per cent dried liver substance: STEPHAN and HARRIS, 1953) as shown in Table 1. Comparable amounts of EDTA were administered in the diet and by intubation. Prior to intubation all animals were fasted 5-6 hr, and immediately thereafter diet was available in an effort to provide maximum contact in the stomach between diet and the intubated solution.

Experiment B

Thirty-six litters of male rats were divided into four groups and fed Diet 633 (35 per cent skim milk powder, 18 per cent cerelose, 45 per cent cornstarch and 2 per cent dried liver substance: MCCLURE and FOLK, 1953) *ad libitum* as shown in Table 2. EDTA was administered in the diet, by intubation and by intraperitoneal injection in doses directly proportional to food consumption.

The animals were on Experiment A for 56 days and on Experiment B 61 days. The jaws were autoclaved and the teeth scored by the method of KEYES (1958). The levels of significance were determined by use of Student's "t" test (CHILTON). Incisor pigmentation was scored as previously described (LARSON, 1958a).

RESULTS

Dental caries. As shown in Table 1, all animals in Experiment A developed caries but the number of carious teeth was significantly greater for the groups receiving EDTA than for the controls. All animals developed lesions in the sulci, with more numerous and more extensive lesions in the EDTA animals than in the controls. The most pronounced effect of EDTA however was a highly significant increase in bucco-lingual and approximal lesions.

All animals in Experiment B also developed caries on one or more tooth surfaces (Table 2). The animals receiving EDTA in the diet or by intubation had significantly more caries activity on the bucco-lingual surfaces and in the sulci than did the controls. Although the injected animals developed somewhat more bucco-lingual and sulcal lesions than the controls, the differences were not significant. In addition, these animals showed uniformly less caries activity than the rats receiving EDTA in either the diet or by intubation. The rats receiving EDTA by diet or intubation showed significantly greater caries activity in the approximal enamel areas than in the controls. No such increase was shown for the EDTA injected rats.

Both these experiments indicate that intubated EDTA was associated with an increase in caries whether the animals were fasted prior to intubation as in Experiment A or fed *ad libitum* as in Experiment B.

Data on haemoglobin (Table 1) and incisor pigmentation (Table 2) indicate that EDTA in the diet or by intubation had an adverse effect on iron utilization. While the mechanism of this interference is unknown, CHAN (1956) and FOREMAN and TRUJILLO (1954) have suggested the possibility that certain cations, such as copper and cobalt, which are necessary for iron utilization may be removed by chelation

from the intestinal tract before being absorbed as available ions. Injected EDTA had no significant effect on iron utilization, as evidenced by incisor pigmentation (Table 2). This finding is in keeping with our earlier report (LARSON, 1958a) on a small group of rats in which the incisor pigmentation and terminal haemoglobin of sodium EDTA injected rats were essentially the same as those of the controls.

In Experiment A the animals were trio-fed the same quantity of diet and the weight gains were the same for each group. In Experiment B the animals were fed *ad libitum* throughout and as reported earlier (HARRIS, 1955) EDTA caused a reduction in food consumption which resulted in lower weight gains.

DISCUSSION

The purpose of the present study was to seek information on the mechanism by which EDTA potentiates caries activity in rats. Interpretation of the results of the administration of EDTA by various routes in these studies demands consideration of the possible role of coprophagy. Thus the increase in caries activity associated with EDTA given by intubation may be ascribed to its re-circulation to the oral cavity by coprophagy. This possibility is strengthened by the observation of FOREMAN, VIER and MAGEE (1953) that 80-95 per cent of intubated EDTA appears in rat faeces within 24 hr. On the other hand there was markedly less caries activity in the rats receiving EDTA by intraperitoneal injection than by intubation. The fact that injected EDTA passes rapidly out of the vascular system to mix with approximately 90 per cent of the body water and 95-98 per cent is excreted in the urine within 6 hr (FOREMAN *et al.*, 1953) would indicate that, under the test conditions employed in this study, there would be less likelihood of entrance of EDTA into the oral cavity. The principal sources would be salivary secretion and contamination of food and faeces with urine. The data by FOREMAN *et al.* (1953) on the fate of intubated and injected EDTA are thus complementary to the possible effect of coprophagy on the development of caries following the administration of EDTA by various routes as in the present study.

The problem of coprophagy in the rat (BARNES *et al.*, 1957) is of particular importance in experimental caries research. Recently KEYES (1960) and FITZGERALD and KEYES (1960) have demonstrated the role of coprophagy in the transmission of the "cariogenic microflora" between rodents. These workers have also pointed out the necessity of distinguishing between direct and physiological effects of dietary variables and effects due to modifications in the oral and intestinal microflora in the interpretation of experimental caries studies. MCCLURE (1960) has called attention to the re-circulation of faecal inorganic phosphate and mentions the possibility of an oral effect of phosphate made available by the hydrolysis of sodium phytate in the lower intestinal tract of rats. The increase in dental caries associated with multiple housing of animals likewise may be a result of increased opportunity for coprophagy or for the contamination of the diet with excreta (MCCLURE and FOLK, 1955).

It is of interest to observe that in contrast to EDTA, caries potentiation by dehydroacetic acid either by oral or extra-oral routes (ZIPKIN and MCCLURE, 1957,

1958) cannot be explained easily by coprophagy since only about 15 per cent of ingested dehydroacetic acid appears in the urine and faeces (SHIDEMAN, WOODS and SEEVERS, 1950).

In addition to the problem of coprophagy it should be noted that caries activity may be affected by variable eating habits. Thus in Experiment A the pair-fed control rats were without food for long periods of time because they quickly consumed the limited amount of diet offered. They developed no bucco-lingual lesions in contrast to the typically high incidence of such lesions as found in *ad libitum*-fed animals in previous studies (STEPHAN and HARRIS, 1953; HARRIS, 1955). On the other hand, the rats receiving EDTA had diet available at all times and more frequent eating provided more contacts between the diet and the teeth. This fact may be partially responsible for the greater caries activity associated with variable feeding regimens observed previously (HARRIS, 1955; LARSON, 1958b; MUHLER, 1955), and will be dealt with more extensively in a separate report.

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THE PREPARATION OF THIN SECTIONS OF DENTAL ENAMEL

J. H. FREMLIN*, JUNE MATHIESON* and J. L. HARDWICK†

*Department of Physics, University of Birmingham, and

†Department of Preventive Dentistry, University of Manchester, England

Abstract—A technique for the preparation of sections of enamel for optical microscopy down to $3\ \mu$ thickness is described. The sections are ground by hand while being supported by being fixed to a microscope slide by a proprietary self-polymerizing adhesive. The factors which influence the optimal thickness of sections for microscopical examinations are discussed and the importance of finely polishing both surfaces of the section is stressed, especially for phase contrast and interference microscopy.

INTRODUCTION

IN THE microscopical examination of a transparent tissue it is desirable to use a thin section to avoid confusion between the layer under observation and optical artifacts arising from other layers. Two criteria must be considered in determining the optimal thickness. The first is optical; there is no need to reduce the thickness below the depth of field of the objective to be used, and for a transparent material it is essential not to reduce the thickness so far that there is insufficient difference of optical density to permit a distinction to be made between different parts of the specimen. The use of such techniques as fluorescence, phase-contrast or interference microscopy may change this limitation but cannot eliminate it.

The second criterion concerns the structure of the tissue. The section should be thin enough to eliminate unwanted structures but sometimes it may be desirable to ensure that it is thick enough for the observer to determine the relation between the layer of the specimen observed and the layers above and below.

The basic unit of human dental enamel is the prism with a diameter of $6-7\ \mu$. In the long axis of the prism a fairly regular structural pattern occurs, which in the case of cross-striations repeats at intervals of a few microns. The roughly cylindrical form and the differences in the refractive indices of these normal structures make it particularly important to avoid the superimposition of several layers. If the lower surface of a thick specimen is focussed upon, it is seen through a set of cylindrical distorting lenses. If its upper surface is examined a number of optical artifacts in the form of badly focussed and distorted line images of the light source due to the underlying layers may be observed, together with a variety of complex diffraction patterns due to the periodic structure. A skilled observer may be able to recognize and discount many of these appearances but they necessarily make his efforts to observe genuine fine structures difficult.

Taking first the structural criterion, the optimal thickness for enamel should be 6–7 μ or less for sections parallel to the prisms and perhaps 3–4 μ or less for transverse sections. It may be advantageous to reduce the thickness of sections parallel to the prisms still further to remove the top and bottom sides of the prisms for study of their internal structure. The useful lower limit for optical microscopy is set by the optical properties of the specimen and by the method of microscopical examination to be used. Certainly the thickness of the section must be at least a micron to obtain an observable differentiation of different enamel structures by any of the present techniques. Thus the diamond-knife methods developed by FEARHEAD (1960) to cut 0.25 μ sections for electron-microscopy are of little use for work with visible light.

With transmitted or polarized light, differentiation between enamel structures becomes increasingly difficult with thicknesses below 7 μ . Interference or phase-contrast microscopy can be used satisfactorily at least down to 3 μ thickness but are much more sensitive to irregularities of the surfaces of the section: for these techniques a fine polish of both surfaces is therefore essential.

The technique to be described was developed, therefore, not to produce the thinnest possible sections, but to fill an important gap in the range of section thicknesses available.

PRINCIPLES OF THE METHOD

The chief difficulty in grinding thin sections of enamel for optical microscopy arises from its combination of hardness and brittleness. Many abrasives will cut freely through enamel but, when used under the wrong conditions, may rip out large pieces of a prism or of a series of contiguous prisms.

It is not easy therefore to grind a free unsupported section to a thickness of less than 20–30 μ . Below this the section will need support and, to be most effective, the support must be at least as hard and as rigid as the enamel itself. In the early part of this investigation this was not appreciated and polystyrene or methyl methacrylate was used as a support. Such supporting media did not prevent the fragmentation of enamel, because they deformed under local pressure to an extent greater than the brittle enamel could tolerate.

Subsequently it was found that the section obtained adequate support if it were mounted on a glass microscope slide, which has a hardness and rigidity similar to that of enamel, by the thinnest possible layer of a proprietary self-polymerizing colourless and transparent adhesive, Eastman adhesive No. 910. This polymerizes under pressure at room temperature so that there is no problem of evaporating a solvent through the narrow gap between impervious glass and nearly impervious tooth. The section does not need to be heated. After setting the refractive index of the adhesive is 1.495, which is close to that of immersion oil, and it does not develop strains which interfere with polarizing microscopy. If so desired, the section may be examined *in situ* on the microscope slide used for its support.

This form of support enables sections to be prepared down to about 7–8 μ throughout the whole tooth and with some specimens it is possible to prepare sections

over more limited areas of enamel down to $3\ \mu$ before the specimen fragments. Sections of dentine can frequently be prepared over limited areas down to $1\ \mu$, if so desired.

With such thin sections optical artifacts due to surface irregularities become important because at least one surface of the specimen will lie within the depth of field of an high power objective. It is therefore necessary to grind finely and polish both surfaces of the section so that they are as nearly as possible optically flat. This is achieved by hand grinding and polishing with graded powders down to a particle size of $0.25\ \mu$, on a flat surface of a suitable material.

TECHNIQUE

A thick section of the tooth is cut in the desired plane on a conventional diamond-loaded wheel to a thickness of 1–1.5 mm.

Grinding and polishing of the first surface

One surface of the specimen is ground by hand on plate glass with Aloxite powders (graded aluminium oxide abrasive, Carborundum Co. Ltd., Manchester, 17) dispersed in water. When one section is being prepared, a single grade, Aloxite 700, is used. When several are to be prepared at the same time, it is usually quicker to use a coarser grade (400) at first and then to continue with the 700 grade powder. Whenever grinding or polishing is continued with a finer grade of abrasive, care must be taken to avoid transferring any of the coarser abrasive into the finer abrasive. The section and the operator's hands should be meticulously clean before going on to the finer grade of abrasive. For the same reason fine abrasive or polishing powders must be protected from contamination by airborne dust particles by being covered when not in use.

The surface of the section is then polished with a suspension of $6\text{--}3\ \mu$ particle size diamond dust in a non-drying base (marketed as Dialap Compound by Power Tools Ltd., Henly Street, Birmingham, 11) on a resilient surface (e.g. Micro-cloth, Shandon Scientific Co. Ltd., Cromwell Place, London S.W.1).

The polishing is completed with $0.25\ \mu$ particle size diamond dust dispersed in water on a flat surface of pitch.

The pitch surface is prepared by melting the pitch, incorporating 3–4 per cent by volume of turpentine, filtering through butter muslin and then pouring on to a flat brass plate to cool. Before the actual polishing the diamond dust should be rubbed lightly but thoroughly into the pitch surface with a small piece of Micro-cloth.

The final polishing should be performed with a regular and continuous rotating action and the section should not be left standing on the surface, to prevent the surface from being deformed. The most suitable degree of wetness of the surface is quickly found by practical trial.

Fixation of the section to the microscope slide

The microscope slide is thoroughly cleaned in chromic acid for several hours, well washed in water and carefully dried. Except in hard water areas, tap-water is

usually preferable to distilled water for washing, being less likely to leave a greasy film.

The section is dried by holding it for a few seconds in a warm blast of air from a hair drier.

A small drop of the adhesive is placed on the slide and the section placed on it polished face downwards. A thin sheet of Cellophane, Pliofilm or paper is placed on the section to prevent the operator's thumb becoming stuck to the slide and heavy pressure is immediately applied with the thumb to squeeze out as much adhesive liquid as possible. After about a minute, the pressure is maintained by a heavy weight on a soft pad. The joint often feels firm after less than a minute but it is wise to maintain pressure overnight to give time for the adhesive to polymerize completely.

Grinding and polishing of the remaining surface

If the microscope slide with the attached section is left in water for a long time the adhesive comes away from the glass but not from the tooth section. For this reason the specimen attached to the microscope slide is now ground down on plate glass with abrasive powders dispersed in a good vacuum or motor oil instead of water to a thickness of 20–30 μ with the 400 grade Aloxite, and to 7–10 μ with the 700 grade Aloxite. It is advisable to use only light pressure and to examine the thickness of the section frequently in the later stages.

The section is smoothed, its thickness being reduced by a further 2 μ , using successively finer grades of Dialap compound (6 μ particle size, followed by 3 μ and then 1 μ particle size) on Micro-cloth. The final polish may be obtained on pitch as before or on cork. Water can be used as a dispersing agent with the pitch at this stage if the polishing is done quickly but glycol is safer; oils dissolve the pitch. A very light oil (e.g. Dialap fluid) can be used with cork; it makes the polishing slower but safer. The final polishing usually reduces the section by a further 1–2 μ .

DISCUSSION

For most purposes the finally polished specimen can be examined satisfactorily still fixed to the original microscope slide. If necessary, the section can be removed from the slide by immersion for a few hours in water. In this case the thin coating of adhesive adheres to the section but does not appear to alter the histological appearances.

The technique uses expensive material but, if contamination by dust particles or by abrasive particles of larger particle size is avoided the same abrasive and polishing materials can be used repeatedly. The adhesive is costly and has a shelf-life of only a few months but little is used for any individual preparation and, if stored in the cold, its shelf-life is prolonged.

Other polishing and abrasive materials can be used if of a suitable particle size, providing that they are slightly harder than the enamel (e.g. Moh hardness 7 and above). Jeweller's rouge gives a satisfactory polish; unfortunately it discolours the dentine and the dentinal tubules become permanently filled with the rouge particles.

The technique demands meticulous attention to detail although it does not

necessitate great manual skill and can be mastered in a few hours. The first surface of a section may be ground and polished in under 10 min. The grinding down and polishing of the second surface is much more time-consuming as more material has to be removed and care has to be taken, especially in the later stages, to check repeatedly the thickness of the section before advancing to the next stage of the technique. With reasonable care there is little risk of damage until the sections are reduced to 7–10 μ thickness. During the final polish the use of high pressure or a long working time are dangerous; it is desirable therefore to polish only as much as is necessary for the method of observation proposed. Two complete sections can usually be prepared in about 7 hr actual working time.

Grinding on a resilient surface such as Micro-cloth for the stage before the final polishing is slower but is safer and produces fewer scratches than plate glass. It tends to leave the harder enamel slightly raised above the dentine; this makes the final fine polish on pitch or cork quicker and easier.

The importance of a good polish is illustrated in the photomicrographs. Fig. 1 represents a smooth but unpolished surface which appeared quite satisfactory under ordinary illumination but which is here photographed using phase-contrast. Figs. 2 and 3 represent polished specimens similarly photographed. It will be noted that in Figs. 2 and 3 there are almost no cloudy areas like those which impair the definition in Fig. 1.

Acknowledgements—We are indebted to the Medical Research Council for financial support, and to Messrs. Degenhardt for the use of a Zeiss Photomicroscope.

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FIG. 1. Transverse section of enamel prisms from young adult tooth. Thickness approx. $7\ \mu$, unpolished. Phase-contrast, $\times 800$.

FIG. 2. Transverse section of enamel prisms from young adult tooth. Thickness approx. $3\ \mu$, polished. Phase-contrast, $\times 800$.

FIG. 3. Longitudinal section of enamel prisms from young adult tooth. Section thickness approx. $5\ \mu$, polished. A—an artifact due to an embedded diamond dust particle. Phase-contrast, $\times 800$.

THE PREPARATION OF THIN SECTIONS OF DENTAL ENAMEL

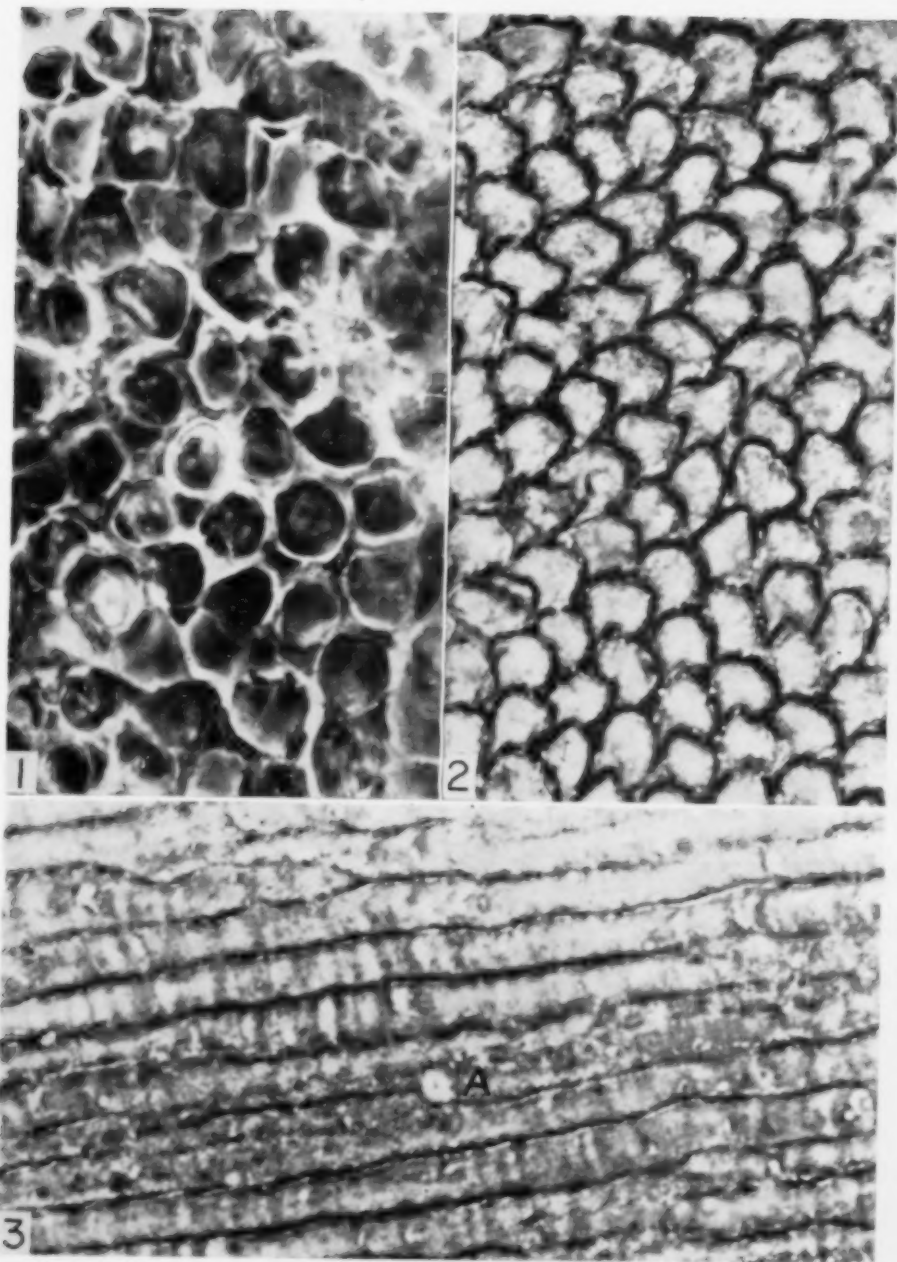


PLATE I

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SHORT COMMUNICATION

PRESENCE OF ENAMEL-COVERED CUSPS IN RAT MOLARS

L. B. JOHANNESSEN

425 East Las Flores Drive, Altadena, California, U.S.A.

ADDISON and APPLETON (1921) reported that the occlusal surfaces of cusps of molar teeth in albino rats are devoid of enamel. Although the histological appearance of the young enamel organ was similar to that of ordinary mammalian teeth, differences were noted soon after the onset of enamel formation. These investigators were able to distinguish between formative and non-formative cells. Functional ameloblasts of the first molars were $21\ \mu$ or longer whereas the non-functional cells measured only about $7\ \mu$.

MELLANBY (1939) and HOFFMAN and SCHOUR (1940) reported also that the summits of the cusps of rat molars are not covered with enamel.

The availability of newer methods of study has permitted a reinvestigation of the occlusal surfaces of cusps of unerupted mandibular first molars in albino rats, and has revealed that the summits of these cusps are not always devoid of enamel.

A total of seven male and seven female albino rats (Holtzman) was killed at the age of 17 days. The unerupted mandibular first molars were removed, dehydrated in alcohol, and embedded in epoxy resin. Subsequently, mid-coronal ground sections were prepared ($95\text{--}105\ \mu$ thick) by removing equal amounts from the lingual and buccal surfaces. The sections were exposed to Grenz rays (20 kV 15 MA 5 min) and photomicrographs were made of the microradiographs. An example is illustrated in Fig. 1, which demonstrates four cusps of the mandibular first molar in a 17-day-old albino rat. The abbreviations M, CM, CD and D refer to the mesial, centro-mesial, centro-distal and distal cusps, respectively.

Enamel was present on part of the occlusal surface of the mesial cusp in most of the specimens. The location and the quantity of enamel differed from one rat to the other, as well as from the molar on the right side to that on the left side in the same animal. This is illustrated in Figs. 1 and 2, which represent the left and right mandibular first molars, respectively, in the same rat.

No enamel could be found on the occlusal surface of the centro-mesial cusp in any of the rats.

As in the case of the mesial cusp, the summit of the centro-distal cusp was covered with enamel in most of the molars, and this enamel was also unevenly distributed.

A uniform layer of enamel, continuous with the enamel on the sloping sides of the distal cusp, was observed in every first molar of this sample (Figs. 1 and 4).

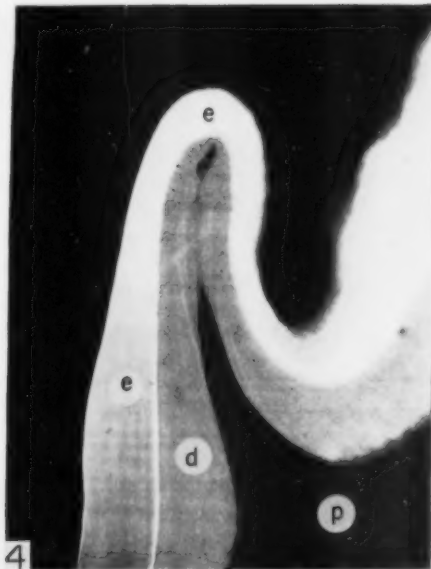
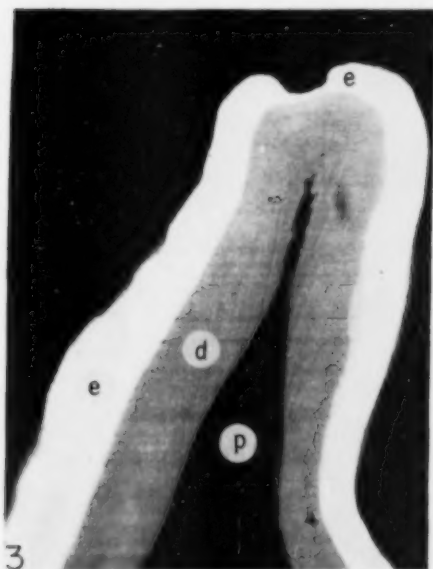
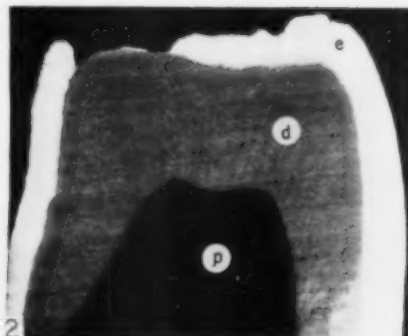
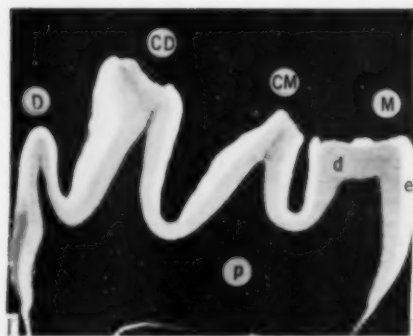
The apparent absence of enamel on the occlusal surfaces of rat molar teeth, previously reported by other investigators, may be explained by differences in material as well as in method. ADDISON and APPLETON (1921) and MELLANBY (1939) used decalcified material and were, therefore, unable to observe enamel on their sections. Although HOFFMAN and SCHOUR (1940) prepared ground sections of undecalcified specimens, they did not embed the rat molars before grinding. The enamel fractures easily under such circumstances, especially from the occlusal aspects of the cusps. Furthermore, because most of the molars used in their study had been subjected to attrition, any enamel that may have been present prior to clinical eruption would have been worn away.

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PRESENCE OF ENAMEL-COVERED CUSPS IN RAT MOLARS



All figures are photomicrographs of microradiographs of mid-coronal ground sections of mandibular first molars in 17-day-old albino rats. Abbreviations: M, mesial cusp; CM, centro-mesial cusp; CD, centro-distal cusp; D, distal cusp; d, dentine; e, enamel; p, pulp.

FIG. 1. Overall aspect of molar. $\times 12$.

FIG. 2. Mesial cusp. $\times 71$.

FIG. 3. Centro-distal cusp. $\times 31$.

FIG. 4. Distal cup. $\times 43$.

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SPIROCHAETAL ABSCESSSES IN HAMSTER CHEEK POUCH

A. A. RIZZO, E. G. HAMPP and S. E. MERGENHAGEN

National Institute of Dental Research, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Md, U.S.A.

THE CONSPICUOUS presence of spirochaetes in ulceromembranous gingivitis and in deep infected periodontal pockets has warranted many studies on the pathogenicity of oral spirochaetes. In a recent extensive work, the literature was reviewed and new data on the pathogenic potential of three species of oral spirochaetes were reported (HAMPP and MERGENHAGEN, 1961). Acute and chronic abscesses were obtained with pure cultures of these organisms in both rabbit and guinea pig skin. In order to extend these results to another species and to oral tissue itself, tests for susceptibility to spirochaetal infection were made on the hamster cheek pouch wall.

The techniques of growing, harvesting and counting spirochaete cells were similar to those described previously (HAMPP and MERGENHAGEN, 1961). Golden hamsters weighing approximately 100 g were anaesthetized with Nembutal and both pouches of each animal washed with sterile saline and stretched over a wide mounting board. Injections of *Borrelia buccalis* and of small oral treponemes were made under 10 \times magnification, with a 30 gauge long-bevelled needle, in the mucosa of the pouch near the blind end. Approximately 3×10^5 bacterial cells suspended in 0.01 ml of fluid were introduced, giving rise to a bleb about 3 mm in diameter. Organisms were usually inoculated in one pouch and either sterile or spent medium inoculated in the contralateral pouch. Gross and microscopic examinations were performed on specimens taken at different time intervals after inoculation.

Within 24 hr well-defined abscesses (Fig. 1) developed in all of the pouch walls inoculated with organisms, whereas no lesions appeared at sites given medium only. The lesions showed haemorrhage and hyperaemia peripherally and contained a central core of thick, yellowish-white exudate. After reaching maximum severity in 24-48 hr, without ulcerating, the lesions gradually regressed. Within 6-8 days the purulent exudate had become absorbed, so that only hyperaemia and induration remained. After several weeks the lesions were represented by small scars. Histologically, the lesions (Fig. 2) were comparable with those in skin described previously (HAMPP and MERGENHAGEN, 1961). The well-delineated inflammatory exudate contained neutrophils, macrophages, spirochaetes and debris (Fig. 3). Dark-field examination of material recovered from 24 hr lesions showed actively motile spirochaetes. The observation that small inocula of pure cultures of oral spirochaetes can cause typical abscesses in an oral tissue suggests that such organisms may play

a similar role in diseased oral sites where they are often extremely abundant. These and previous experimental findings (HAMPP and MERGENHAGEN, 1961) in two other animal species indicate that these organisms may have a consistent degree of pathogenicity in mammalian species.

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SPIROCHAETAL ABSCESSSES IN HAMSTER CHEEK POUCH

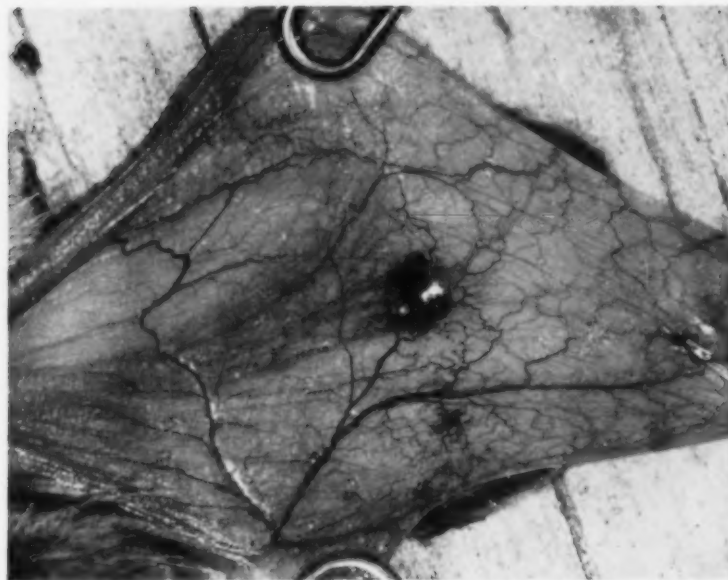


FIG. 1. Macroscopic view of experimental hamster cheek pouch abscess obtained with small oral treponeme (72 hr duration).

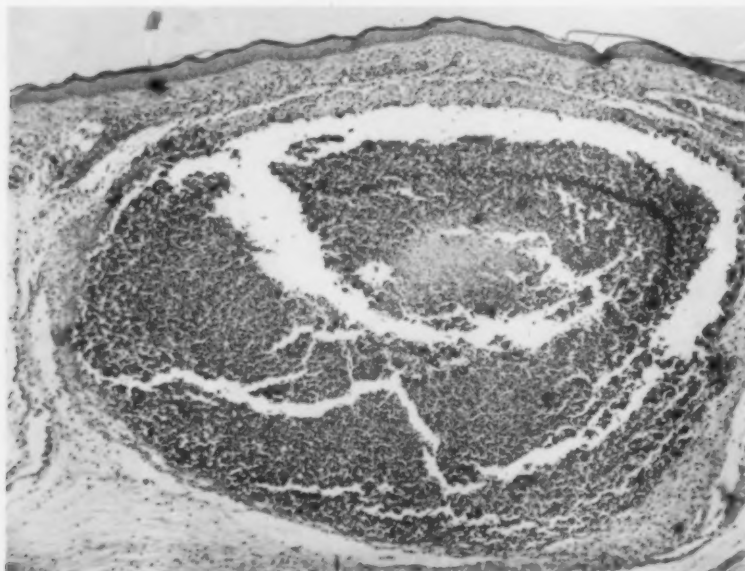


FIG. 2. Photomicrograph of 48 hr abscess obtained with *Borrelia buccalis*. Haematoxylin and eosin. $\times 33$.

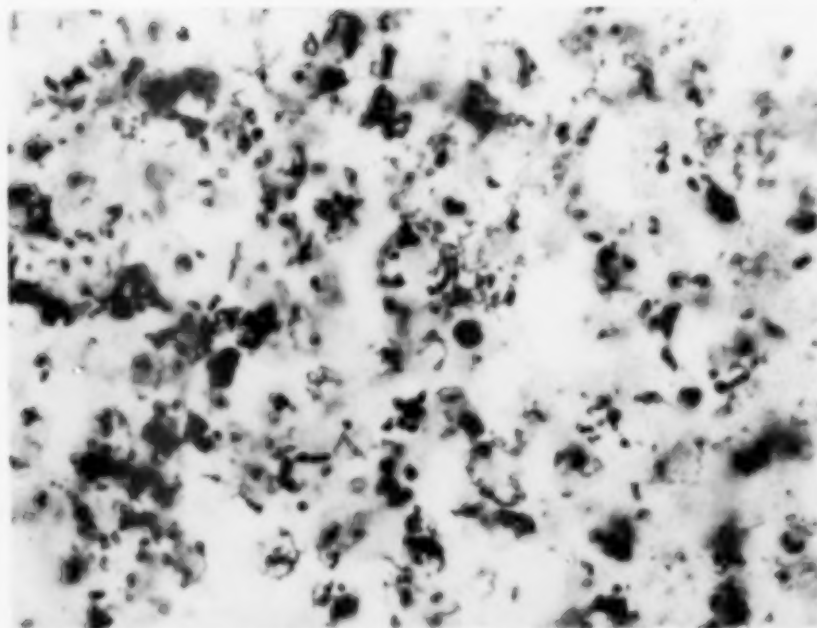


FIG. 3. High-power photomicrograph of same specimen as Fig. 2 showing organisms in the tissue. Warthin Starry. $\times 1250$.

BOOK REVIEWS

S. BLACKMAN: *An Atlas of Dental and Oral Radiology*. Williams & Wilkins, Baltimore, 1960. 371 pp., \$15.00.

ACCORDING to the Preface it is the hope of the author "that this book will meet increasing needs and demands in dentistry and radiology to provide a reference book for the fundamental interpretation of dental conditions. In addition it should stimulate a better understanding of dental pathology." The atlas consists of three parts. Part I deals with the eruption and development of the teeth in sections covering radiographic appearances of normal anatomical structures, normal tooth development as well as developmental aberrations, anomalies in tooth formation and calcified odontomes. In this part salivary calculi are also included. The second part illustrates inflammatory and traumatic lesions of the teeth and jaws under the headings of tooth concussion, dental caries, periapical disease, marginal periodontitis, internal root resorption, osteitis and osteomyelitis, fractures of the teeth, fractures of the jaws and facial bones, pathological fractures and finally the maxillary antrum. Part III describes cysts and new growths involving teeth, jaws and soft tissue in the following sections: cysts of the jaws, tumours of the jaws and mouth, soft tissue tumours of the oral cavity and lastly systemic diseases.

The book presents good photographic reproductions of dental radiographs. This is particularly true in regard to the intra-oral films. Some of the extra-oral pictures have been reduced rather much and are difficult to interpret. The text is—as expected in such an atlas—very short. It is found as introductions to the different sections, summarizing pertinent classification and some explanation of the pathology. Under such conditions a close connexion is expected between the introduction and the figures with their brief legends. This is however not always the case, as in the section on salivary calculi, which appear divided into dental calculi and salivary stones. Not one picture of dental calculus is included in the section. Occasionally some of the statements may be questioned. The first radiographic change in marginal periodontitis is stated to be an irregular widening of the black periodontal shadow around the cervical margins of the teeth. What about changes in the interdental bone crest?

Sometimes there is a tendency on the part of the author to read more in to the pictures than can actually be seen. Thus Figs. 121 and 554 are identical. In the first case in the section dealing with submerged teeth a lower second molar with a follicular cyst is described as being pushed downwards and mesially. In the section on odontogenic cysts the tooth is said to be partially erupted. In Fig. 325 the spreading osteitis from the apex of a lower third molar is described as encroaching upon the mental foramen and the inferior dental canal. The mental foramen is not shown in the picture. In addition, the distinction between chronic dento-alveolar abscess, rarefying osteitis and apical granuloma on the basis of radiographic evidence, appears questionable to the present reviewer.

These remarks should however not overshadow the fact that many of the sections in the atlas are very good and that a wealth of information is presented in the many radiographs. The weakness in this book is in the text, which is not clear and concise enough. Radiography is only a diagnostic aid—even if a very important one—and radiographic interpretation is most meaningful in the light of clinical and laboratory findings. For this reason the inclusion of an Appendix discussing differential diagnostic considerations with references to the atlas and to other publications would greatly enhance the value of this book as a reference volume.

As it stands, this book is a valuable adjunct to any dental library even if it fails to be the ideal reference book on dental and oral radiology.

P. GRØN

Calcification in Biological Systems. Edited by R. F. SOGNNÆS. American Association for the Advancement of Science, Washington, 1960. xiv+511 pp., \$9.75.

THIS book is a collection of papers which, with the exception of the first article on "Calcification in unicellular organisms", were all delivered orally at a Symposium of three sessions held in conjunction with the 125th Annual Meeting of the A.A.A.S. in Washington, D.C. in December 1958. This Symposium was largely organized by REIDAR F. SOGNNÆS, who, in the Preface, describes the scientific stimuli which inspired the Symposium, and outlines its main theme, i.e. the fundamental mechanism of calcification and mineral deposition in a wide variety of biological systems. A number of important monograph and conference reports concerned with mineralized tissues have appeared in recent years, but these have been largely bone-centred, and have emphasized the contribution of bone to the equilibrium of the body fluids. The present volume presents a refreshing new point of view which should be of particular interest to those who are concerned with the field of oral biology. Emphasis is placed on the elements which contribute to hard tissue formation and calcification *per se*, with equal space and time given to dental, osseous and other more or less specialized forms of mineralization in both lower and higher animal forms. The authors, drawn from many biological disciplines, are authorities on the topics presented, and have given not only an excellent review of knowledge in the field, but also reports of their own recent work. The first group of papers are

concerned with calcification in unicellular organisms and lower animals (molluscs, echinoderms and crustaceans), and in the mineralization of turkey leg tendon and the otolithic organ of the rat. Next, the elements involved in the calcification of dental, osseous and pathological tissues in man are considered, including abnormal tissue calcifications, and deposition of salivary calculi. Two papers deal with experimental induction of osteogenesis and behaviour of bone in tissue culture, while the last four papers are concerned with the structural nature of the inorganic and organic building blocks of skeletal tissue in man, and their relationship. Of particular interest is the final paper of this group, which discusses the preferred orientation of bone crystals with respect to the collagen fibrils and the localization, regulation and inhibition of crystallization.

This volume contains much useful information not readily available elsewhere. It will be a valuable addition to the reference shelf of anyone seriously interested in the problems of mineralized tissues.

D. H. COPP

PAPERS ACCEPTED FOR PUBLICATION

R. L. HARTLES and A. G. LEAVER: Citrate in mineralized tissues—IV. The relation of vitamin D intake and calcium nutrition to the citrate content of the rat femur
(Biochemistry Department, School of Dental Surgery, Boundary Place, Liverpool 7)

A. R. TEN CATE: The distribution of glycogen in the human developing tooth
(Department of Dental Science, Royal College of Surgeons of England, Lincoln's Inn Fields, London W.C.2)

D. J. ANDERSON: Tooth movement in experimental malocclusion
(Physiological Laboratory, Guy's Hospital Medical School, London S.E.1)

M. E. WEAVER, F. M. SORENSON and E. B. JUMP: The miniature pig as an experimental animal in dental research
(Department of Anatomy, University of Oregon Dental School, Portland, Oregon)

M. R. WADDELL: The effect of amino acids and related compounds on the metabolism of saliva
(School of Dental Surgery, Boundary Place, Liverpool 7)

A. D. DIXON: The position, incidence and origin of sensory nerve terminations in oral mucous membrane
(Department of Anatomy, University of Manchester, Manchester 13)

T. R. DIRKSEN, M. F. LITTLE, B. G. BIBBY and S. L. CRUMP: The pH of carious cavities; The effect of glucose and phosphate buffer on cavity pH
(School of Aerospace Medicine, Brooks Air Force Base, Texas)

M. BRÄNNSTRÖM: The elicitation of pain in human dentine and pulp by chemical stimuli
(Department of Dental Histopathology, The Royal School of Dentistry, Stockholm, Sweden)

S. M. WEIDMANN: Uptake and retention of fluoride by teeth of animals under experimental fluorosis
(Biological Research Unit, School of Dentistry, University of Leeds, Leeds)

A. I. DARLING, K. V. MORTIMER, D. F. G. POOLE and W. D. OLLIS: Molecular sieve behaviour of normal and carious human dental enamel
(Dental School, University of Bristol, Lower Maudlin Street, Bristol 1)

O. BACKER DIRKS, B. HOUWINK and G. W. KWANT: The results of 6½ years of artificial fluoridation of drinking water in the Netherlands. The Tiel-Culemborg experiment
(Laboratory of Microbiology, University of Utrecht, Utrecht, Netherlands)

R. J. GIBBONS and S. S. SOCRANSKY: Intracellular polysaccharide storage by organisms in dental plaques; Its relation to dental caries and microbial ecology of the oral cavity
(Forsyth Dental Infirmary, 140 The Fenway, Boston 15, Massachusetts)

Vol.
5
1961

CURRENT PAPERS IN ORAL BIOLOGY

Honorary Editor: MAURICE V. STACK

CLASSIFICATION of titles has been revised following the listing of 2000 *Current Papers* in issues comprising the first three volumes of the *Archives*. Headings for Fluoride Studies (5) and Periodontology (9) replace those formerly covering material on Genetics and Microbiology.

Abbreviated postal addresses are included, where known, in the present *Current Papers Section* in order to facilitate contact with (principal) authors.

Types of research covered by the present twelve headings are as follows:

- (1) **Anatomy.** Structures and relationships of hard and soft tissues.
- (2) **Normal Histology.** Studies using (electron) microscopy, histochemistry, microradiography, autoradiography, etc.
- (3) **Physiology.** Functions of oral and dental tissues and salivary glands.
- (4) **Biochemistry.** Components and reactions, solubility studies, crystallography.
- (5) **Fluoride Studies.** Caries surveys, prophylaxis, experimental and toxicology.
- (6) **Histopathology.** Pathology investigated by techniques as in (2) above.
- (7) **Experimental Pathology.** Induced structural and functional changes.
- (8) **Dental Caries.** Publications bearing directly upon dental decay processes.
- (9) **Periodontology.** Tissues in health and disease, including relevant microflora.
- (10) **Epidemiology and Clinical Studies.** Distribution, incidence and investigational treatment of oral disorders affecting groups of cases.
- (11) **Materials and Techniques.** Advances in dental research methods.
- (12) **Miscellaneous.** Contributions not directly classifiable under the above.

The following list covers names of those 185 periodicals from which more than one title has been quoted among the first 2000 *Current Papers*; abbreviations follow those recommended in the *World List of Scientific Periodicals* or, for journals not listed therein, *World Medical Periodicals* (WHO & UNESCO, 1957).

Acta anat.
Acta med., Acad. Sci. hung.
Acta morph., Acad. Sci. hung.
Acta odont. scand.
Acta otolaryng., Stockh.
Acta paediat. Stockh.
Acta path. microbiol. scand.
Acta physiol. scand.
Acta stomat. belge
Acta stomat. hellen.
Actualités odontostomat.
Amer. J. Anat.
Amer. J. clin. Nutr.
Amer. J. clin. Path.
Amer. J. Orthodont.
Amer. J. Path.
Amer. J. phys. Anthropol.
Amer. J. Physiol.
Amer. J. publ. Hlth
Amer. J. Surg.
A.M.A. Arch. industr. Hlth

A.M.A. Arch. Path.
A.M.A. Arch. Surg.
An. esp. Odonto-Estom.
Analyst
Analyt. Chem.
Anat. Anz.
Anat. Rec.
Anesthesiology
Angle Orthodont.
Ann. Soc. belge Méd. trop.
Ann. Stomat., Roma
Ann. N.Y. Acad. Sci.
Ann. Otol., St. Louis
Ann. R. Coll. Surg. Engl.
Arch. Anat., Leningrad
Arch. Biochem. Biophys.
Arch. gen. Psychiat.
Arch. histol. japon.
Arch. int. Pharmacodyn.
Arch. ital. Biol. orale
Aust. dent. J.

CURRENT PAPERS IN ORAL BIOLOGY

- Aust. J. biol. Sci.*
Biochem. J.
Biochim. biophys. Acta
Biol. Neonat.
Brit. dent. J.
Brit. J. exp. Path.
Brit. J. Nutr.
Brit. J. plast. Surg.
Brit. med. J.
Bull. Northw. Univ. dent. Sch.
Bull. Tokyo med. dent. Univ.
Cah. odonto-stomat.
Canad. med. Ass. J.
Chin. J. Stomat.
Clin. chim. Acta, Amst.
C.R. Acad. Sci., Paris
Čsl. stomat.
Czas. stomatol.
Dent. Practit. Rec.
Dent. Progr.
Dtsch. Zahn-, Mund- u. Kieferheilk.
Dtsch. Zahnärztl. Z.
Dtsch. Z. ges. gerichtl. Med.
Endocr. japon.
Exp. Cell Res.
Fed. Proc.
Fogorv. Szle
Gig. i Sanit.
Growth
Harefuah
Helv. odont. Acta
Hum. Biol.
Int. dent. J., Lond.
Jap. J. Physiol.
J. All-India dent. Ass.
J. Amer. dent. Ass.
J. Amer. med. Ass.
J. Anat., Lond.
J. appl. Physiol.
J. Bact.
J. belge Radiol.
J. biol. Chem.
J. Bone, Jt Surg.
J. Canad. dent. Ass.
J. dent. Ass. S. Afr.
J. dent. belge.
J. Dent. Child.
J. dent. Med.
J. dent. Res.
J. exp. Med.
J. Geront.
J. Histochem. Cytochem.
J. Indian Acad. Dent.
J. invest. Derm.
J. Japan. stomat. Soc.
J. lab. clin. Med.
J. mol. Biol.
J. nerv. ment. Dis.
J. Nihon Univ. Sch. Dent.
J. Nutr.
J. Okayama med. Ass.
J. oral Surg.
J. Osaka odont. Soc.
J. Osaka Univ. dent. Soc.
J. Path. Bact.
J. Pediat.
J. Periodont.
J. Physiol.
J. prosth. Dent.
J. Radiol. Electrol.
J. R. micr. Soc.
J. Ultrastruct. Res.
Kokubyo Z.
Lancet
Med. J. Aust.
Microchem. J.
Mikroskopia
Minerva derm.
Minerva stomat.
Nature, Lond.
Naturwissenschaften
New Engl. J. Med.
N. Y. St. dent. J.
N.Z. dent. J.
Norske Tandlægeforen. Tid.
Obstet. Gynec.
Odont. Revy
Odont. Tidskr.
Odont. urug.
Oral. Surg.
Öst. Z. Stomatol.
Oto-rhino-laring. ital.
Panninerva Med.
Parodontologie, Zürich
Plast. reconstr. Surg.
Pract. oto-rhino-laryng., Basel.
Proc. Nutr. Soc.
Proc. Nutr. Soc. Lond.
Proc. R. Soc. Med.
Proc. Soc. exp. Biol., N. Y.
Proc. zool. Soc., Lond.
Publ. Hlth. Rep., Wash.
Q. natl. dent. Ass.
Rass. trim. odontoiat.
Rev. Assoc. Odont. Argent.
Rev. belge Sci. dent.
Rev. brasil. Odont.
Rev. dent., Santiago
Rev. franç. Odonto-Stomat.
Riv. ital. Stomat.
Schweiz. med. Wschr.
Schweiz. Mschr. Zahnheilk.
Science
Sech. physiol. J., USSR
Shigaku
Shikwa Gakuho
S. Afr. med. J.

- | | |
|--------------------------------|---|
| <i>Sov. zdrav. Kirgisia</i> | <i>Tokyo dent. Coll. Bull. oral Path.</i> |
| <i>Stain Tech.</i> | <i>Trans. R. Sch. Dent., Stockh. & Umed</i> |
| <i>Stoma, Heidelb.</i> | <i>U.S. armed Forces med. J.</i> |
| <i>Stomatologie, Liège</i> | <i>Vestn. rentgenol. radiol.</i> |
| <i>Stomatologia, București</i> | <i>Vjschr. naturf. Ges. Zürich</i> |
| <i>Stomatologiya, Moskva</i> | <i>Vopr. med. Khim.</i> |
| <i>Stomatologiya, Sofia</i> | <i>Vrach. Delo</i> |
| <i>Suom. Hammaslääk. Toim.</i> | <i>Zahnärztl. Praxis</i> |
| <i>Surg. Gynec. Obstet.</i> | <i>Z. Anat. Entw. Gesch.</i> |
| <i>Svensk Lakartidn.</i> | <i>Z. Hyg. InfektKr.</i> |
| <i>Svensk tandläk. Tidskr.</i> | <i>Z. physiol. Chem.</i> |
| <i>Tandlägebladet</i> | <i>Z. Zellforsch.</i> |
| <i>Tijdschr. Tandheelk.</i> | |

I. ANATOMY

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BAUME, L. J. and DERICHSEILER, H. (1961) *Oral Surg.* **14**, 347-362.
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(Tandläkarhögskolan, Stockholm 3)
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2006. Persistence of taurodontism in modern races and its possible relationship to pyramidal molar roots. [French]
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2007. Relation between radiographic appearance of the temporomandibular joint and some occlusal features [in children aged 8-13].
BURLEY, M. A. (1961) *Brit. dent. J.* **110**, 195-200.
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2010. Physiological [arch] spacing in relation to carpal maturation.
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2011. Anatomical characteristics of jugal teeth of European Castoridae. [French]
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2017. Observations on malformed teeth. [Japanese]
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2019. Development of the palate in the rabbit. [Japanese]
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2021. Deviations in vertical development of the dentition. [German]
SCHWARZ, A. M. (1960) *Fortschr. Kieferorth.* **21**, 358-381.
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2022. Muscle development and function. [Norwegian]
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2. NORMAL HISTOLOGY

2023. Epithelia in the dento-gingival junction.
ALLDRITT, W. S. (1961) *Dent. Practit. Rec.* **11**, 213-223.
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2024. Nature of enamel organic matrix.
BERKE, J. D. (1961) *N. Y. St. dent. J.* **27**, 59-66.
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2025. Sucrose retardation of acid etching in dental enamel: An electron microscopic study.
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2026. Structure of salivary glands in relation to their electron microscopy. [German]
FERNER, H. (1961) *Dtsch. zahnärztl. Z.* **16**, 128-142.
(Histol. Inst., Univ., Homburg/Saar)
2027. Factors in tooth transplantation. [English]
FLEMING, H. S. (1961) *Parodontologie, Zürich* **15**, 11-32.
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2028. Electron microscopy of normal dentine of deciduous teeth. [Japanese]
FUJINO, S. (1960) *J. Osaka odont. Soc.* **23**, 775-794.
2029. Age changes in the parotid glands. [Russian]
GAIBOV, A. G. (1960) *Zdrav. Tadzhik.* (4), 56-59.
2030. Parotid compartment.
GAUGHAN, G. R. L. (1961) *Ann. Otol., St. Louis* **70**, 31-50.
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2031. Vascular supply in relation to root formation on cheek teeth of the mouse.
GAUNT, W. A. (1961) *Acta anat.* **43**, 116-136.
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2032. Innervation of lingual salivary glands.
GÓMEZ, H. (1961) *Anat. Rec.* **139**, 69-76.
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2033. Root canal diameter measurement. [French]
GREEN, E. N. (1961) *Stomatologie, Liège*, **4**, 212-231.
(Dent. Sch. Miami, Fla.)
2034. Cementum histochemistry. [German]
HAIM, G. (1961) *Dtsch. zahnärztl. Z.* **16**, 71-80.
2035. Histochemistry of succinic dehydrogenase in gingival tissues.
KAWAKATSU, K., MORI, M. and KISHIRO, A. (1960) *Dent. Bull. Osaka Univ.* **1**, 79-87.
(32 Joancho, Kitaku, Osaka)
2036. Electron microscopy of enamel structure, with reference to tufts and cuticle. [French]
LENZ, H. (1961) *Stomatologie, Liège* **4**, 189-211.
(Lehrgebiet Biol. u. Anthropol., Tech. Univ., Berlin-Dahlem)
2037. Quantitative histochemistry of hamster tooth buds: Alkaline phosphatase and lactic dehydrogenase.
NUKI, K. and BONTING, S. L. (1961) *J. Histochem. Cytochem.* **9**, 117-125.
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2038. Clinical aspects of innervation of the teeth. [Swedish]
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2039. Structure of the alveolar socket. [Russian]
PAKALNS, G. (1960) *Izv. Acad. Nauk Latv. SSR* (12), 121-128.
2040. Nuclear sex determination from oral mucosal smears. [Hungarian]
PASTINSZKY, I. (1961) *Fogorv. Szle* **54**, 33-40.
(Széher ú. 78, Budapest)
2041. Molecular sieve behaviour of dental enamel.
POOLE, D. F. G., MORTIMER, K. V., DARLING, A. I. and OLLIS, W. D. (1961) *Nature, Lond.* **189**, 998-1000.
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2042. Sialic acid-containing mucins in bovine submaxillary and rat sublingual glands.
QUINTARELLI, G., TSUIKI, S., HASHIMOTO, Y. and PIGMAN, W. (1961) *J. Histochem. Cytochem.* **9**, 176-183.
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2043. Fluorescence microscopy of penetration of carcinogenic hydrocarbons into oral tissues.
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2044. Electron microscopy of enamel and dentin of teeth of the [sand shark] *Odontaspis* (Selachii).
SASSO, W. da S. and SANTOS, H. de S. (1961) *J. dent. Res.* **40**, 49-57.
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2045. Dentine canals of the canine teeth of *Cervus elaphus*. [German]
SCHMIDT, W. J. (1961) *Dtsch. zahnärztl. Z.* **16**, 337-343.
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2046. Histochemistry of carbonic anhydrase with reference to osteoclasts.
SIMASAKI, M. and YAGI, T. (1960) *Dent. Bull. Osaka Univ.* **1**, 89-98.
(32 Joancho, Kitaku, Osaka)
2047. Odontoblasts: Vacuoles and inclusions.
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(Good Samaritan Hosp., W. Palm Beach, Fla.)

CURRENT PAPERS IN ORAL BIOLOGY

2048. Histochemistry of rodent salivary glands—I. Hydrolytic enzymes; II. Polysaccharides.
TANI, T. (1960) *J. Osaka Univ. dent. Soc.* **5**, 213-230; 231-248.
2049. Recruitment in internal enamel epithelium as a tooth germ growth factor.
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3. PHYSIOLOGY

2050. Histochemistry of rat salivary glands after response to ACTH, cortisone and DOCA.
[Japanese]
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2051. Effect of nicotinic acid on unconditioned reflex function of salivary glands. [Russian]
DEMITCHEV, A. P. (1960) *Fiziol. Zh. SSSR* **46**(5), 561-564.
(Inst. Psychiatry, Moscow)
2052. Temporomandibular joint distortion due to disturbances in the masticatory system. [Dutch]
DERKSEN, I. (1961) *Tijdschr. Tandheelk.* **68**, 77-104; 172-185.
(Stadhouderslaan 66, Soestdijk, Utrecht)
2053. Mitotic activity in pilocarpine-stimulated salivary glands of rats.
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2139. Bacteriology and histochemistry of plaques from caries-active and caries-resistant individuals. [German]
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2153. Pathogenicity of microorganisms in dental calculus. [Japanese]
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2167. Clinical and histological study of the effect of hydrocortisone upon the oral cavity in disease
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2168. Pathogenesis of micrognathia. [German]
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MÜLLER, W. A. and HARTENSTEIN, H. (1960) *Dtsch. med. Wschr.* **85**, 879-882.
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DENTINE APPPOSITION IN THE MANDIBULAR FIRST MOLARS OF ALBINO RATS

L. B. JOHANNESSEN*

Forsyth Dental Infirmary and Harvard School of Dental Medicine,
Boston, Massachusetts, U.S.A.

Abstract—Dentine formation was studied in the mandibular first molars of 264 albino rats (Holtzman Co.), equally distributed as to sex. Thirty-two animals were used to establish the onset of mineralization at the sites of measurements by means of vital staining with alizarin red S. Sixteen rats were divided in two groups, killed on the twentieth and twenty-third day, to test the reliability of the weaning line (21-day weaning) as a reference zone. The remaining 216 animals were divided into two groups. Group 1 animals were injected with a 0.2% solution of NaF at the age of 31, 41 and 61 days to produce three timed calciotraumatic dentine responses. The dose of F at each injection was 20, 20 and 10 mg F per kg of body wt., respectively. The increments of dentine in these rats were studied up to the age of 91 days. Group 2 animals, divided in three sub-groups, were injected at the same age as the experimental ones with physiologic saline solution and killed when 41, 61 and 91 days of age. The injections of saline did not result in calciotraumatic lines. The deposits of dentine were measured on comparable mid-coronal ground sections.

The largest increments per day were observed prior to weaning, namely 13.8, 14.6 and 10.2 μ , on the cervical mesial, distal, and on the mid-occlusal surfaces, respectively. Subsequently, between 21 and 41 days, approximately 7.3 μ of dentine (per day) was deposited on the mid-occlusal surface and 6.7 μ (per day) on the distal cervical surface. The formation of dentine in the latter area had apparently subsided in 22–33 per cent of the rats prior to the age of 61 days because no evidence of the 61 day NaF injection could be observed. However, daily increments of 3.5 μ of dentine accrued on the distal cervical surface between 41 and 61 days in those rats that did show the 61 day NaF line. All the rats continued deposition of dentine on the mid-occlusal surface beyond the ages of 41 and 61 days, but the daily increments between 41 and 61 days were only 3.8 μ in the NaF injected rats as opposed to the 5.1 μ observed in the controls. This deceleration was only temporary, and the mid-occlusal dentine deposit (21–91 days) at age 91 days did not differ significantly from that observed in the saline injected animals. The rate of dentine formation was not associated with the increments in body weight. The magnitude of the dentine deposit and the size of the tooth appeared to be unrelated and there were no statistically significant sex differences. Persistence of the 21 day weaning line and of the induced calciotraumatic responses made it possible to obtain longitudinal data on dentine deposition. Because of the large individual variations in dentine widths at weaning, and because these variations increased in magnitude with age, it is suggested that future investigations of dentine formation should be planned as longitudinal studies.

INTRODUCTION

SKELETAL SHAPE and size are attained and controlled by two basic processes, namely bone apposition and bone resorption. Since these two processes occur simultaneously, it is difficult to study the patterns, rhythms and magnitude of bone apposition during early development.

* Present address: 425 E. Las Flores Drive, Altadena, California.

Dentine, on the other hand, is not ordinarily subjected to remodelling resorption although it has many characteristics in common with bone. Rat incisor teeth have, therefore, been used in many investigations to discover some of the factors that influence the rate of dentine matrix formation and its subsequent mineralization. The incisors of these rodents wear and grow continuously throughout the life span of the animals, and their dentine forms and mineralizes at the rate of approximately 16μ per day. The results observed in studies of dentine formation of rat incisors must be interpreted on the basis of this life-long and daily apposition of 16μ .

Unlike the incisors, the molar teeth of the rat complete most of their growth in the immature animal and remain in function long afterward. Accordingly, the dentine in the molar teeth provides a permanent record of mineral metabolism in contrast to the incisors, where dentine is constantly worn away and is completely replaced by new dentine.

This permanent metabolic record is used in the present study to identify disturbances in the apposition of dentine at specific ages, and to measure the linear increments from such zones as the dentino-enamel junction to an early or late lesion, or from any of these reference lesions or zones to the dentino-pulpal junction. The timing and quantity of these increments indicate when and how much dentine is deposited in different parts of the tooth.

The following three types of metabolic disturbances were used in the present study: the vital staining of dentine by means of alizarin red S prior to weaning; the lesion produced on the day of weaning without experimental intervention; and post-weaning lesions induced by timed injections of NaF.

Apparently HOFFMAN and SCHOUR (1940) made the only quantitative study of dentine apposition in molar teeth of rats. Three animals were used as controls, and the remaining sixty animals were injected with alizarin red S at various age levels. The experimental period started at birth and terminated as late as 500 days of age for some of the rats. The animals had been obtained from four different sources, and their sex was not specified. Certain findings of HOFFMAN and SCHOUR will be considered in a subsequent section of this report.

MATERIALS AND METHODS

In the present study a total of 264 Sprague-Dawley descendant albino rats (Holtzman Co.) were used. The animals were equally distributed as to sex. Immediately after birth, each of the thirty-three litters was reduced to four male and four female pups. Purina rat laboratory chow and tap water were available *ad libitum* throughout the experimental period. The rats were divided into three main groups (Table 1) for the following purposes: to determine the onset of the mineralization of dentine in various parts of the molar tooth (pre-weaning period); to test the reliability of the weaning line as a timed reference zone (weaning period); and to study the post-weaning increments of dentine in the young albino rat (post-weaning period).

Group 1 (pre-weaning) consisted of four litters. Each litter was divided into four pairs of one male and one female pup. On the third, fifth, sixth and tenth day

of age, respectively, one pair of rats from each litter was injected intraperitoneally with a 2% solution of alizarin red S (100 mg alizarin red S per kg of body wt.). The animals were killed at the age of 21 days.

TABLE 1. PROTOCOL OF ALIZARIN RED S AND NaF INJECTIONS IN 264 ALBINO RATS

Group	No. of animals		Age at injection and material used (days)	Age when killed (days)
	Males	Females		
1	4	4	Alizarin red S (100 mg/kg)	
	4	4	3	
	4	4	5	21
	4	4	6	21
	4	4	10	21
2	4	4	No injection	
	4	4	—	20
3	12	12	Saline	
	20	20	31	
	20	20	31 41	41
			31 41 61	61
				91
	56	56	NaF	
			31 41 61 (20 mg F/kg) (20 mg F/kg) (10 mg F/kg)	91

Group 2 (weaning) was composed of two litters. From each litter, two males and two females were killed before weaning, namely at the age of 20 days. The remaining eight animals were killed 2 days after weaning, at the age of 23 days.

Group 3 (post-weaning) consisted of 108 male and 108 female rats, all weaned when 21 days of age. Fifty-six animals of each sex were injected intraperitoneally with a 0.2% solution of NaF at the ages of 31, 41 and 61 days, utilizing dosages of 20, 20 and 10 mg F per kg of body wt., respectively. These rats were killed when they were 91 days old. Physiologic saline solution was administered in the same manner to the 104 control rats. The latter were divided into three groups, and killed at the age of 41, 61 and 91 days, respectively.

Following sacrifice of the animals by ether inhalation, the mandibular first molars were removed and mechanically cleaned. The greatest buccolingual crown diameter was measured with a Starrett micrometer, and the teeth were placed in individual bottles containing absolute alcohol.

Subsequently, rows of eight to ten molars of similar buccolingual crown size were embedded in epoxy resin (Hysol) by means of steel matrices. Comparable mid-coronal ground sections, 95–105 μ thick, were prepared by removing equal amounts

from the buccal and lingual surfaces. The grinding was accomplished on a specially constructed grinding machine that permitted manual control of the vertical as well as the horizontal advance of the section. Predetermined amounts as small as $3-4 \mu$ could be removed at a time. After grinding, the sections were cleared in xylene and mounted in Permout.

Tracings outlining the external enamel surface, the pulpodentinal surface, and the dentino-enamel border were made of each mid-coronal section by means of a Bausch and Lomb Tri-Simplex microprojector at standardized magnification of 46.2 (Fig. 1). The weaning line and other reference zones were also outlined on the tracings, when present.

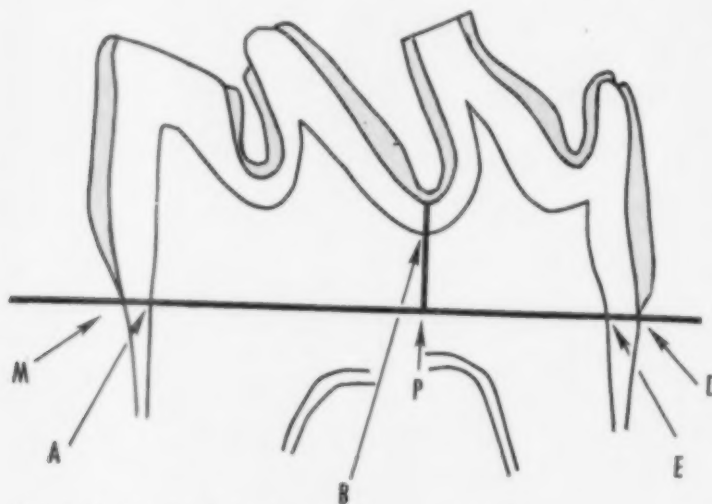


FIG. 1. A tracing of the mid-coronal section of the mandibular first molar of a 21-day-old rat, showing landmarks used in this study. The lines in the tracing have been accentuated to permit photographic reproduction.

A straight line ($M-D$) was drawn on each tracing to connect the mesial (M) and distal (D) cervical dentino-enamel junctions. The intersection of the mesial pulpodentinal contour line and the $M-D$ line was called point A , the corresponding distal point was named E . The dimensions $M-A$ and $D-E$ represented the mesial and distal cervical dentine widths, respectively. The width of the mid-occlusal dentine ($F-B$) was measured from point F to the pulpodentinal surface on the perpendicular $F-P$. Point F was defined as the deepest point on the dentino-enamel border at the base of the central occlusal groove. All measurements on the tracings were done with a Starrett vernier caliper. The standard deviation for the error of measurement (0.5 mm) as well as for the tracing error (1.2 mm) had been calculated in a previous study of rat molar crown morphology (JOHANNESSEN, 1961) by the following formula:

$$\text{S.D. error} = \left(\frac{\text{Sum of differences}^2}{2n} \right)^{\frac{1}{2}}$$

The standard deviation of the error of tracing was determined by tracing the same tooth on two separate occasions. On each tracing a total of twenty-three dimensions were measured twice. Based on the mean differences of the twenty-three dimensions in Tracing 1 and those in Tracing 2, the standard deviation of the tracing error could be calculated. The standard deviation of measurement error was obtained on the basis of differences between ten independent measurements of the twenty-three dimensions on one of the tracings.

In order to report the data in actual dimensions (microns), the measurements were divided by 46.2.

FINDINGS

General findings will be considered first. The animals in Group 1 did not suffer any loss of weight due to the single injection of alizarin red S. The location of the alizarin red S lines in the dentine demonstrated that the dentine adjacent to the central occlusal groove mineralized on the fourth day of life, whereas the cervical dentine on the mesial as well as on the distal aspect of the crown commenced to mineralize on the ninth day. These findings confirmed the chronology of the mandibular first molar crown development previously described by MELLANBY (1939). The weaning line could not be observed in the dentine of the rats in Group 1 (killed on the day of weaning), nor could a weaning line be observed in the dentine of those rats in Group 2 killed prior to weaning. In contrast, the 21 day weaning response was found in the dentine in the molars of all rats killed at age 23 days, and also in the molars of animals killed at subsequent age levels (Group 3). It was, therefore, concluded that the weaning line could be used as a reference zone.

The dentine in the molar teeth from the saline injected rats in Group 3 did not show identifiable lesions in response to the injections. However, the administration of NaF in the sample of fifty-six males and fifty-six females resulted in sharp, easily observed, calciotraumatic responses that were used to measure post-weaning increments of dentine. The injections of NaF were followed by intraperitoneal abscesses in every instance. Although the abscesses healed uneventfully in 6-8 days, the animals did not gain weight at the same rate as did the controls (Table 2). Furthermore, the second injection at 41 days of age, using the same dosage as in the first injection at 31 days, resulted in the death of five males and sixteen females within 2 hr. Consequently, the dosage was reduced to 10 mg F/kg for the third injection at 61 days. No fatalities occurred after decreasing the dosage.

The observation of the onset of dentine mineralization at the sites of measurements, and the reliability of the weaning line as a reference zone made it possible to utilize all rats in Groups 1 and 3 for the purpose of measuring increments in the dentine formation prior to weaning. The apposition of dentine during the pre-weaning period will, therefore, be described first.

Table 3 presents the total width of dentine on the mesial, mid-occlusal and distal molar crown surfaces at 21, 41, 61 and 91 days in male and female rats, respectively. At the time of weaning, the average amount of dentine accrued on the mid-occlusal surface was 173 μ , and on the distal cervical surface 175 μ , in both sexes. Smaller

TABLE 2. BODY WEIGHTS OF ALBINO RATS IN RELATION TO NaF AND SALINE INJECTIONS

Males		Age (days)	Injection (group)	Females	
No.	Mean \pm S.D. (g)			No.	Mean \pm S.D. (g)
40	82.0 7.3	27	Saline	40	73.1 8.8
56	74.8 9.5		NaF	56	67.5 6.8
40	112.0 9.2	31*	Saline	40	98.0 8.5
56	102.5 10.4		NaF	56	92.7 6.7
40	132.2 9.2	34	Saline	40	113.2 9.1
56	116.6 10.2		NaF	56	103.1 7.9
40	156.3 10.0	37	Saline	40	130.1 8.9
56	139.1 11.7		NaF	56	118.4 7.8
40	187.5 11.9	41*	Saline	40	149.3 9.4
56	168.1 12.6		NaF	56	140.1 8.1
40	205.9 12.7	44	Saline	40	157.0 8.8
51	174.5 14.1		NaF	40	140.6 8.2
40	221.8 9.8	47	Saline	40	162.0 8.9
51	190.5 14.4		NaF	40	147.2 9.4
40	247.2 13.3	51	Saline	40	171.7 9.9
51	215.0 12.5		NaF	40	159.5 10.2
40	286.6 25.9	61*	Saline	40	190.6 12.6
51	254.1 20.5		NaF	40	181.2 12.2
20	303.9 25.7	64	Saline	20	199.2 12.5
51	264.7 23.4		NaF	40	185.4 13.2
20	314.4 26.6	67	Saline	20	202.2 13.1
51	276.0 23.0		NaF	40	188.8 13.5
20	328.0 30.2	71	Saline	20	205.9 11.9
51	291.6 23.7		NaF	40	195.7 11.4
20	357.3 31.2	81	Saline	20	214.0 11.9
51	326.3 25.1		NaF	40	207.6 9.0
20	376.5 33.0	91	Saline	20	221.8 12.3
51	347.5 30.3		NaF	40	216.2 15.8

* Day of injection.

amounts of dentine, namely 164 μ in males and 166 μ in females, were apposed on the mesial cervical surface. The difference between the amount of dentine accrued on the mesial and that deposited on the distal cervical surface from 9 to 21 days of age was statistically significant (C.R.=4.0 for males and 2.9 for females), but, as

could be expected, the two dimensions were correlated at better than the 1 per cent level of significance.

TABLE 3. TOTAL DENTINE WIDTHS IN MANDIBULAR FIRST MOLARS OF ALBINO RATS IN RELATION TO NaF AND SALINE INJECTIONS

Age (days)	Site*	Injection (group)	Males			Females		
			No.	Mean \pm S.D. (μ)		No.	Mean \pm S.D. (μ)	
21	M-A	None	72	164	17	57	166	17
	D-E	None	99	175	19	80	175	19
	F-B	None	106	173	19	83	173	17
41	M-A	Saline	8	313	19	11	316	18
		NaF	21	316	19	8	305	28
	D-E	Saline	9	298	19	11	303	21
		NaF	47	311	22	27	320	23
	F-B	Saline	9	326	17	11	324	11
		NaF	49	307	36	29	331	43
61	M-A	Saline	15	391	28	14	391	26
		NaF	7	383	30	5	352	44
	D-E	Saline	19	380	23	18	385	25
		NaF	34	387	30	24	393	26
	F-B	Saline	19	419	36	18	426	30
		NaF	46	383	41	29	411	51
91	M-A	Saline	16	413	22	13	404	25
		NaF	44	400	25	27	402	28
	D-E	Saline	17	430	30	14	415	25
		NaF	49	406	28	29	415	32
	F-B	Saline	17	491	49	14	474	41
		NaF	49	458	41	29	489	45

* Site: M-A and D-E: mesial and distal cervical surface, respectively;
F-B: mid-occlusal surface.

In terms of daily increments, the rates of dentine deposition were found to be 13.8, 14.6 and 10.2 μ on the mesial, distal and mid-occlusal surface, respectively. However, relatively large individual variations in the total amounts of dentine present at weaning were observed, suggesting that dentine widths at any age after weaning should be measured from the weaning line and not from the dentino-enamel border.

Therefore, the increments of dentine during the post-weaning period were listed in Table 4 as total amounts of dentine deposited from 21 to 41, 61 and 91 days, on

the distal and mid-occlusal surfaces. The rates of dentine deposition henceforth described were calculated from the results presented in this table unless noted otherwise. Furthermore, the increments of dentine in the control animals were determined cross-sectionally from the differences observed between the mean values of the three different control groups, whereas the deposits of dentine in the NaF injected rats were determined longitudinally.

TABLE 4. INCREMENTS OF DENTINE FROM 21 TO 41, 61 AND 91 DAYS IN MANDIBULAR FIRST MOLARS OF ALBINO RATS

Age (days)	Site*	Injection (group)	Males			Females		
			No.	Mean \pm S.D. (μ)		No.	Mean \pm S.D. (μ)	
21-41	D-E	Saline	9	132	15	11	137	13
		NaF	47	134	17	26	137	14
	F-B	Saline	9	140	18	11	153	18
		NaF	49	138	23	29	155	32
21-61	D-E	Saline	17	198	17	16	206	19
		NaF	26	215	24	21	216	24
	F-B	Saline	19	244	30	17	253	24
		NaF	46	213	32	29	233	43
21-91	F-B	Saline	17	310	49	14	301	41
		NaF	49	293	30	29	312	39

* Site: D-E: distal cervical surface; F-B: mid-occlusal surface.

During the period from 21 to 41 days, the daily increments of dentine on the distal cervical surface were approximately the same in the NaF and in the saline injected rats (6.6μ in males and 6.8μ in females). The apposition of dentine continued in this area after 41 days of age. However, the calciotraumatic response of the 61 day NaF injection was absent in 22 and 33 per cent of the males and females, respectively. Evidently, dentine deposition on the cervical surface had subsided in these animals before they reached the age of 61 days. Daily increments in dentine from 41 to 61 days could be calculated for the twenty-six males and twenty-one females that exhibited the 61 day NaF injection response and it was found that these increments were 3.7 and 3.4μ in males and females, respectively. Daily increments (41-61 days) could not be calculated for the control rats since there was no way to determine how many of these actually deposited cervical dentine up to the age of 61 days.

For the same reason, daily increments in cervical dentine could not be calculated for the period 61-91 days in the control rats. The total mean increase in dentine

accrued during this time was 50 μ in males and 30 μ in females and the corresponding values for the NaF injected animals were 31 and 24 μ , respectively. The injections of NaF did not affect cervical dentine deposition to extents that approached statistical significance; neither were any statistically significant sex differences observed in the amounts of dentine that accrued on the cervical surface.

The rate and magnitude of dentine apposition on the mid-occlusal surface during this post-weaning period presented a different picture. Between 21 and 41 days the male deposited an average of 7.0 and the female 7.6 μ of dentine per day. These increments were slightly larger than those observed on the cervical surface for the same period. The injections of NaF did not decelerate dentine apposition during this period. However, for the 41-61 day period, when the male control rats deposited 5.2 μ and the female controls deposited 5.0 μ of dentine per day, the NaF injected animals accrued only 3.7 and 3.9 μ , respectively, per day. The difference between the NaF and the saline injected groups in amounts of dentine deposited on the mid-occlusal surface from 21 to 61 days was statistically significant for the males (C.R.=3.8) but not for the females (C.R.=2.0).

The formation of dentine continued on the mid-occlusal surface between 51 and 91 days. The calciotraumatic response in dentine due to the 61 day injection of NaF was observed in all rats in the NaF group. The mean total increase in dentine width in the NaF group was 80 and 79 μ in males and females, respectively. During this same 61-91 day period, the corresponding values for the saline injected rats were 76 μ for males and 48 μ for females. Furthermore, when the total 21-91 day deposits of dentine in the two groups were compared, the difference between the NaF and the saline injected rats was not statistically significant. Because NaF or another "marker" substance had not been injected on the day prior to sacrifice, it could not be determined if all the rats apposed dentine up to the age of 91 days. Consequently, daily increments of dentine on the mid-occlusal surface between 61 and 91 days were not calculated.

Because the injections of NaF affected the gains in body weight, at least temporarily, and because the increments in dentine as well as body weight decreased in magnitude with age in all the rats, it was decided to determine if increments in dentine and increments in body weight were correlated. Correlation coefficients were calculated for mid-occlusal dentine as well as for the distal cervical dentine increments and the corresponding increments in body weight between the ages of 31-41, 41-61 and 61-91 days. Only low degrees of association were found (r ranged from -0.111 to +0.382) and none of the correlation coefficients were statistically significant.

The mesiodistal cervical crown size ($M-D$) and central internal crown height ($F-P$) have already been studied for the molars used in the present study (JOHANNESSEN, 1961) and scattergrams were made of the mid-occlusal width of dentine and the central internal crown height at 21, 41, 61 and 91 days, as well as of the distal cervical dentine width and cervical mesiodistal crown dimensions at the ages of 21 and 41 days. No association was observed at weaning or at subsequent ages between tooth size and dentine width.

DISCUSSION

Alizarin red S and NaF are both useful for the purpose of producing timed reference zones in the dentine. In addition, the naturally occurring weaning line serves to differentiate between pre- and post-weaning dentine formation.

The findings regarding pre-weaning apposition of dentine on the mesial cervical surface differ from those reported by HOFFMAN and SCHOUR (1940). While these investigators calculated the daily increments of dentine on this surface to be 9.33μ (from 7 to 14 days) and 8.69μ (from 14 to 20 days), larger increments, namely 13.8μ , were found in the present study. However, HOFFMAN and SCHOUR calculated the initial increments of dentine from the seventh day and not from the tenth, although they had apparently recognized that the crowns in their rats were not completed at the cemento-enamel junction until the tenth day. Consequently, if the initial rates of 9.33 and 8.69μ are adjusted to the timing of crown development, the increments approximate 16μ per day. In view of the fact that HOFFMAN and SCHOUR measured the dentine width along the curved dentinal tubules, one can expect their findings to be of greater magnitude than those of the present study in which measurements were made along a straight line (*M-D*).

The general pattern of dentine formation in mandibular first molars of albino rats followed certain gradients. First of all, the early increments of dentine were larger than the subsequent ones and, secondly, the dentine was not deposited in equal amounts in all areas of the crown (Fig. 2). When one considers that these teeth are subjected to attrition even before weaning and the amounts of dentine deposited between the ages of 21 and 91 days in areas not directly subjected to attrition, it becomes apparent that factors other than occlusal wear probably determine the "final" dentine dimensions in the molar teeth of rats.

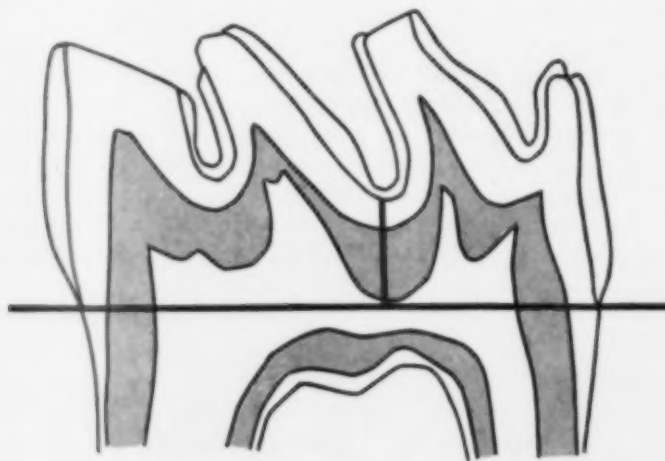


FIG. 2. A tracing of the mid-coronal section of the mandibular first molar of a 21-day-old albino rat. The shaded area represents dentine that will be deposited between 21 and 91 days.

Investigations are planned to determine if the response of actively forming mineralized tissues, such as the dentine in molars of young rats, to metabolic disturbances is related to their stage of development. In addition, it should be established whether or not the increments of dentine actually occur daily.

Because the dentine in mandibular first molars of albino rats begins mineralization at about the time of birth while the magnitude of dentine apposition decreases with age and is different on various pulpal surfaces, the albino rats are well suited for experimental studies of factors influencing the apposition of dentine.

In view of the relatively large individual variations in the width of dentine already present at the time of weaning, it is apparent that dentine should be measured from the weaning line rather than from the dentino-enamel border.

Acknowledgement—This investigation was carried out during the tenure of a Special Fellowship from the National Institute of Dental Research, United States Public Health Service.

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HEREDITARY LIMITATIONS OF THE INFECTIOUS AND TRANSMISSIBLE NATURE OF EXPERIMENTAL DENTAL CARIES

S. ROSEN, H. R. HUNT and C. A. HOPPERT

Department of Zoology and Chemistry, Michigan State University,
East Lansing, Michigan, U.S.A.

Abstract—We have confirmed that dental caries is an infectious and transmissible disease in albino rats. However, the transmission of this disease was significantly demonstrated only with rats which were genetically susceptible. Rats bred for susceptibility to caries showed an increase in caries activity after depressing the penicillin-sensitive flora and reinoculating with faeces. Rats bred for resistance to caries did not show a significant increase in caries activity after depressing the penicillin-sensitive flora and reinoculating with faeces. This confirms the view that the genotype of a rat is one factor in determining its resistance to dental caries. Faeces from either caries-susceptible or caries-resistant rats induced caries to the same extent in susceptible rats. This indicates that caries-resistant rats were naturally exposed to a caries-active flora, but failed to develop this disease extensively.

INTRODUCTION

A RECENT REPORT (KEYES, 1960) shows that caries can be induced in caries-inactive animals by exposure to caries-active animals. Consequently, we have asked ourselves the question, "Are there genetic differences between our caries-susceptible and caries-resistant lines of rats (HUNT, HOPPERT and ERWIN, 1944), or is the existence and non-existence of caries in these lines due exclusively to the presence or absence of a cariogenic oral flora?"

Studies by two groups of investigators (SHAW, 1960; SHAW and GRIFFITHS, 1960; ROSEN, HUNT and HOPPERT, 1961) show that when newborn rats of a caries-resistant line are nursed by a caries-active female, the young rats remain resistant. Conversely, a newborn rat from a caries-susceptible strain remains susceptible when nursed by a caries-resistant mother. It has also been reported (SHAW, 1960; SHAW and GRIFFITHS, 1960; STEPHAN and HARRIS, 1954) that placing different strains of rats into the same cage does not alter the caries experience expected for a particular strain.

The experiments reported herein were designed to determine the extent to which caries could be transmitted to an animal which was presumed to be either genetically susceptible or resistant to this disease.

EXPERIMENTAL METHOD

The caries-susceptible stock used descended from a cross between our original susceptible strain and Osborne-Mendel rats secured from the National Institutes of Health. These rats are designated the OMS line. The caries-resistant animals have

been maintained in our laboratory for 23 years. A modified coarse particle Hoppert-Webber-Canniff caries-test diet was fed (HOPPERT, WEBBER and CANNIFF, 1932). (This was composed of rice ground so that 1-2 per cent was retained on a 20 mesh screen, 66 per cent; whole powdered milk, 30 per cent; alfalfa, 3 per cent, and iodized salt, 1 per cent). From birth to weaning, the rats were given the above diet containing in addition, 500 units of penicillin per gram (Antibiotic Feed Supplement—Source of Penicillin, Chas. Pfizer and Co., Terre Haute, Indiana).

Littermates were then divided into four experimental groups as follows:

(1) These rats were given the caries-test diet without penicillin and the teeth were swabbed with 0.05% sterile yeast extract water. This group is designated as control rats in Table 1.

(2) The rats remained on the caries-test diet containing 500 units of penicillin per gram and were swabbed as in (1) above. This group is listed under rats with penicillin in diet in Table 1.

TABLE 1. THE EFFECT ON MEAN CARIES SCORES OF INOCULATION WITH FAECES FROM SUSCEPTIBLE (OMS) AND RESISTANT RATS
(Superscripts indicate numbers of rats)

Caries-susceptible (OMS)					Caries-resistant				
Days on experiment	Control rats	Rats with penicillin in diet	Inoculum		Days on experiment	Control rats	Rats with penicillin in diet	Inoculum	
			Faeces from susceptible rats	Faeces from resistant rats				Faeces from susceptible rats	Faeces from resistant rats
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)
110	19.0 ¹	0.0 ¹	40.0 ¹	52.0 ¹	169	0.0 ²	—	1.5 ²	0.5 ²
110	6.0 ¹	0.0 ¹	30.0 ¹	42.0 ¹	169	2.0 ²	—	1.0 ²	3.5 ²
110	21.0 ²	2.0 ²	28.0 ²	32.0 ²	169	6.0 ¹	—	4.0 ¹	0.0 ¹
					169	0.5 ²	—	0.5 ²	1.0 ¹
84	19.5 ²	0.0 ²	46.5 ²	34.0 ²	84	0.0 ²	0.0 ²	2.5 ²	3.0 ²
84	0.0 ¹	0.0 ¹	46.0 ¹	38.0 ¹	84	0.0 ²	0.0 ²	0.0 ²	0.0 ²
82	12.3 ²	2.5 ²	22.7 ²	20.0 ²	82	0.0 ¹	0.0 ²	0.0 ¹	0.0 ¹
82	7.0 ²	0.5 ²	22.5 ²	13.5 ²	82	0.0 ¹	0.0 ¹	0.0 ¹	0.0 ¹
82	10.5 ²	0.0 ¹	16.3 ²	19.5 ²	82	0.0 ¹	0.0 ¹	0.0 ¹	0.0 ¹
82	15.0 ²	0.7 ²	22.5 ²	14.5 ²	82	0.0 ²	0.0 ¹	0.0 ²	0.0 ¹

(3) The rats were given the caries-test diet without penicillin and the teeth swabbed with a suspension of faeces from a caries-susceptible rat in 1 ml of 0.05% yeast extract water. The animals of this group are listed under faeces from susceptible rats in Table 1.

(4) The same as (3) above, except that the source of faeces was from a caries-resistant rat. This group is listed under faeces from resistant rats in Table 1.

In another experiment (see Table 2), the same procedure was used as described above, but instead of inoculating with faeces a cotton-tipped applicator was used to brush the teeth of either a caries-susceptible or caries-resistant rat, and, immediately thereafter, the same swab was used to brush the teeth of the OMS rats.

TABLE 2. THE EFFECT OF MEAN CARIES SCORES OF INOCULATION WITH SWABBINGS OF TEETH FROM SUSCEPTIBLE (OMS) AND RESISTANT RATS
(Superscripts indicate numbers of rats)

Caries-susceptible (OMS)				
Days on experiment	Control rats	Rats with penicillin in diet	Inoculum	
			Swabbing of teeth from susceptible rats	Swabbing of teeth from resistant rats
(a)	(b)	(c)	(d)	(e)
65	4.5 ²	0.0 ³	14.0 ³	19.0 ³
65	13.5 ³	0.0 ¹	23.5 ³	12.0 ³
65	7.5 ²	0.0 ²	16.0 ²	26.5 ²

Cotton-tipped applicators were used for the swabbings, all of which were continued for 10 consecutive days.

From two to four litters of each strain were used in each experiment. The time for each individual experiment was constant, but the time from one experiment to another ranged from 65 to 169 days (Tables 1 and 2). The starting time was the day of the initial swabbing. When faeces were used as the inoculum, pellets for each experiment were obtained from animals approximately 3 weeks older than the experimental animals. Faecal pellets were secured fresh each day that the animals were swabbed. Animals from the same litter were used as the source of inoculum for any one experiment. Thus, animals kept on the experiment for 110 days received inoculum from a different single litter than those rats kept on experiment for 84 days.

However, in the experiments carried out for 84 and 82 days, both caries-resistant and caries-susceptible rats were used concurrently. Therefore, each of these strains was inoculated with faeces from the same source.

At the termination of the experiment, the rats were sacrificed and scored (Cox *et al.*, 1939). The teeth were hemisectioned with a steel saw in order to evaluate lesions deep in the fissures (KEYES, 1958b).

RESULTS

The data are presented in Tables 1 and 2 and a summarized statistical analysis is given in Table 3. Each caries score listed in Tables 1 and 2 represents the score of from one to the mean of three animals. The data given in a horizontal row were

obtained from one litter. For example, in Table 1 the third horizontal row of numbers under caries-susceptible (OMS) indicates that the rats of that litter were kept on the experiment for 110 days, the mean caries score of the controls was 21.0, the mean score of rats that remained on penicillin was 2.0, the mean score of the OMS rats receiving inoculum from OMS rats was 28.0 and the mean score of the OMS rats receiving inoculum from resistant rats was 32.0. No attempt was made to measure the variability because the data were obtained from experiments performed for varying lengths of time. This condition was taken into account in the statistical treatment of the data given in Table 3. The data from two columns were compared by paired data analysis (SNEDECOR, 1946). The data from Tables 1 and 2 were combined for this analysis.

TABLE 3. PAIRED-DATA ANALYSIS OF RESULTS GIVEN IN TABLES 1 AND 2

Strain of rats	Groups being compared (Letters refer to columns in Tables 1 and 2)	<i>t</i>	<i>P</i>
Susceptible	Control (b) and Susceptible (d)	4.70	<0.01
Susceptible	Control (b) and Resistant (e)	3.79	<0.01
Susceptible	Susceptible (d) and Resistant (e)	0.09	>0.05
Resistant	Control (g) and Susceptible (i)	0.26	>0.05
Resistant	Control (g) and Resistant (j)	1.15	>0.2
Resistant	Susceptible (i) and Resistant (j)	1.15	>0.2

The transmissibility of caries was obvious with the susceptible rats. The caries score was higher in every pairing for these rats receiving inoculum from the OMS rats than in the controls. The difference between these two groups is statistically significant (see Table 3). Furthermore, caries was transmitted when susceptible rats received inoculum from caries-resistant rats. The degree of caries in this group was about the same as in the group receiving inoculum from OMS rats. There was no statistical difference between these two groups (see Table 3).

In contrast to the ease of transmission of caries into the susceptible rats, caries could not be transmitted to any considerable extent by inoculation of faecal material into the resistant rats, although the same effort was made to transmit caries to this strain as to the susceptible strain. Furthermore, resistant strain rats kept on experiment for as long as 169 days failed to show more than a negligible degree of caries. The caries-resistant rats never developed caries to the degree of control susceptibles. That is, caries-susceptible rats with the penicillin-sensitive bacterial flora depressed during their weaning period still developed caries to a moderate extent in contrast with the near-absence of caries in resistant rats, which had the penicillin-sensitive flora depressed but were later infected with faecal inoculation. These data prove that heredity is an important factor in the aetiology of dental caries, at least for our strain of rats, when a coarse particle caries-test diet is used.

DISCUSSION

In a recent publication (KEYES, 1960) it has been suggested that investigators who desire to establish or work with caries-resistant experimental animals should determine the results of using different caries-test diets and find out whether the animals carry a flora with a "cariogenic" potential. This comment is pertinent and all past, present and future claims for genetic caries-resistance must consider the above factors.

The data presented in this paper clearly indicates that hereditary factors influence a rat's capacity to develop caries. This concept of heredity in no way detracts from the importance of environmental agents in the aetiology of dental caries. Micro-organisms are indispensable instruments in caries production. The nature of the diet is also extremely important. If a high fat ration or a finely ground diet are used, caries-resistant rats cannot be easily distinguished phenotypically from caries-susceptibles, as these diets are known to be of "low cariogenicity". If certain micro-organisms are prevented by antibiotics from growing in the oral cavity, or if the rats are raised "germ free", it is impossible to distinguish caries-resistant from caries-susceptible rats as judged by their phenotypes.

However, heredity can limit the production of caries. The experiments reported in this paper show that caries can be transmitted provided that the animal is genetically predisposed to the development of caries. If it is genetically resistant, massive infection with faecal material will not cause a significant increase in caries.

An animal which fails to develop caries only because the necessary micro-organisms are absent from the mouth merely copies resistants, but it does not have the genes for resistance. The environmental copy and the genetic type look alike, but they are different kinds of individuals. Many similar cases are reported in genetic literature.

The extremely caries-resistant line of rats originally developed (HUNT *et al.*, 1944) has declined in resistance (ROSEN *et al.*, 1959). Some animals of this line have been shown to be about as resistant to caries now as Sprague-Dawley rats (KEYES, 1958a) which are generally used as caries susceptibles. The rigorous selection practised prior to 1952 was relaxed, so that the familiar phenomenon of genetic reversion may have accounted for at least a part of the drift toward susceptibility. This could happen because the line was never homozygous for resistance. We are now resuming selection in this line, attempting to improve our methods for detecting cavities. We intend to make the stock as uniform for high resistance as possible.

It has been reported that caries-inactive hamsters became caries-active when they were caged with other caries-active hamsters (KEYES, 1960). The terms caries-inactive and caries-active as applied to these hamsters are probably appropriate. It has not been shown that this species possess genes for resistance, though we suggest that a geneticist might be able to find such genes. It is apparent that certain hamsters possess a "cariogenic" oral flora which can be transmitted to another hamster which does not possess a "cariogenic" oral flora. Although there are several preliminary reports suggesting that there are genes for caries-resistance in hamsters (HODGE *et al.*, 1953; HODGE, JOHANSEN and HEIN, 1955; JOHNSON and HODGE, 1956), a successful line of this type has not been established.

What is the distinction, then, between a caries-inactive and a genetically caries-resistant or immune individual? A caries-inactive individual is one with a low caries index; it may or may not carry genes that retard or prevent the development of caries. If a caries-inactive individual is subjected to an environment which causes it to develop caries in a relatively short time, then it is obviously not genetically caries-resistant. If an individual can withstand this environmental challenge and is able to transmit this trait to at least some of its progeny, then that individual may be termed caries-resistant.

It is conceivable, though not demonstrated, that the hamster species (*Cricetus cricetus*) has no genes for caries resistance. On the other hand, we have presented data which supports the view that we have a line of albino rats which is genetically caries-resistant rather than caries-inactive.

Acknowledgement—We are indebted to the National Institute for Dental Research, National Institutes of Health, Public Health Service, Bethesda, Maryland, for supporting this research by Grant D-367.

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THE INFLUENCE OF SULPHONAMIDE CONES UPON SOCKET HEALING IN THE RAT

A. M. FRANDSEN and J. J. PINDBORG

Royal Dental College, Department of Oral Pathology, and Leo Pharmaceutical
Company, Department of Biology, Copenhagen, Denmark

Abstract—At the age of 60 days, eighty female rats had a first lower molar extracted. They were arranged in groups of ten according to post-operative intervals ranging from 1 to 49 days. In each group eight animals had a sulphonamide cone inserted into the socket, whereas two animals were left untreated as controls. The healing process was studied microscopically by conventional procedures, and polarized light was used to identify further the sulphonamide particles. No inhibition by the cones on fibrous and bony healing of the sockets could be demonstrated. The sulphonamide particles did not evoke any foreign body reaction, nor did they cause inflammatory changes. Particles were present in the socket area 49 days after the extraction, the longest post-operative period examined.

INTRODUCTION

THE TOPICAL application of sulphonamides in the sockets after tooth extraction in humans has been the subject of a considerable number of reports. Several of the sulphonamides have been investigated, applied as pure crystals or powders, or in combination with various vehicles. Opinions differ as to the effects of these procedures. Some investigators report a beneficial effect mainly consisting of the prevention of post-operative infection, specifically the "dry socket", whereas others claim that the sulphonamides act as a foreign body and delay healing. A comprehensive survey of the literature may be found elsewhere (KRASHEN, 1942; OSTRANDER, 1943; OSTRANDER and KERR, 1947) and will not be given here.

The majority of these reports are based on clinical evaluation, and the experimental evidence is less convincing. As the topical application of sulphonamides after tooth extraction is still widely used, and recommended as a means of preventing "dry socket" (KROGH, 1948) and as a precaution when extracting in the presence of acute infections (KROGH, 1951), it was thought desirable to seek information about the effects of these procedures on the processes involved in socket healing.

Only a few investigators have attacked these problems by means of histologic examination. In dogs, OSTRANDER and KERR (1947) medicated the sockets after extraction with various quantities and forms of sulphonamides and examined the healing histologically. They demonstrated that a combination of sulphonamides with eugenol was highly irritating to the tissues. Epithelial healing occurred, although later than normal, but new bone did not form in the socket, which was partly filled with a crystalline material as late as 30 days after the extraction. A 60% sulphathiazole-glycerine ointment did not cause irritation and delay in healing, but the

formation and organization of the blood clot was somewhat disturbed. Dry sulphonamide powder dusted into the socket did not cake and was not retained as a foreign body. After 10 days no crystalline material could be identified.

KEY and BURFORD (1940) implanted sulphonamide crystals in compound fractures of the forelegs in rabbits. By histologic and radiographic examination they could demonstrate no difference in the formation of bone between treated animals and controls.

GLYNN (1941) investigated the effects of topical application of various powdered sulphonamides on the development of granulation tissue and on striped muscle. With regard to granulation tissue he concluded that a slight, barely significant, inhibition of fibroblast proliferation was produced by sulphathiazole and sulphapyridine but not by sulphanilamide. This inhibition was not considered sufficient to contra-indicate the local application of these drugs. Failure of healing after an apicoectomy when a sulphonamide preparation was placed in the bone cavity was reported by GUSTAFSON (1958). Ten years after the apicoectomy a biopsy from the operated area exhibited crystal-like structures, some of which were being resorbed by giant cells. When observed in polarized light these structures showed birefringence. Only a slight lymphocytic infiltration was present, indicating that the preparation was tolerated fairly well by the tissues.

Normal socket healing consists of five distinct but overlapping phases (HUEBSCH *et al.*, 1952): formation of blood clot, organization of the clot by ingrowth of granulation tissue, epithelial closure, formation of woven bone, and reorganization and replacement of woven bone by lamellar bone. Of particular interest as far as uncomplicated healing is concerned is clot formation, clot organization, and initiation of bone formation. Since information as to the influence of sulphonamides on these tissue processes is either lacking or contradictory, it was decided to apply the conditions of a well-controlled animal experiment to these problems.

MATERIAL AND METHODS

Eighty female rats of the Leo strain were weaned at 21 days of age and reared on a stock diet (Standard diet, Leo Pharmaceutical Company). The mandibular left first molar was extracted at 60 days of age, and the rats were arranged in groups according to post-operative intervals (Table 1). The animals were anaesthetized by an intraperitoneal injection of a 1% solution of Nembutal^R (0.8 mg per 100 g body wt.). The extraction technique was as previously described (HEUBSCH *et al.*, 1952). A sulphonamide cone was placed in the distal socket of all the experimental animals, whereas the controls were left untreated.

The rats were sacrificed by decapitation under ether anaesthesia. The heads were skinned and fixed in 10% neutral formalin. They were cut sagittally in the midline and the left mandibles disarticulated. The socket area was decalcified in a mixture of equal parts of 40% formic acid and 20% sodium citrate solution. The specimens were embedded in paraffin, cut buccolingually in serial sections and stained with haematoxylin-eosin and van Gieson's connective tissue stain.

TABLE 1. DISTRIBUTION OF RATS ACCORDING TO TREATMENT AND POST-OPERATIVE PERIODS

Total number of rats: 80

No. of rats in each group	Treatment	Days after extraction
4	Micronized cones	} 1-2-3-4- 7-14-21-29
4	Non-micronized cones	
2	Controls	
10		8 groups

The sulphonamide cones were made of equal parts of sulphathiazole and sulphanilamide, since these are the most widely used of the sulphonamides for topical application. The binding material was starch (*amylum solani*) and the ratio of sulphonamides to starch was 65:30. Half of the treated animals received micronized cones, the other half non-micronized cones. By the micronizing process an attempt was made to procure a uniform size of the sulphonamide particles, well below crystal size.

RESULTS

It was not possible to distinguish any difference in the tissue reactions between the animals treated with micronized cones and those treated with non-micronized cones. Therefore, these two groups will be described as one.

Fig. 1 illustrates a socket from a control animal 24 hr after the extraction. The socket is clearly distinguishable; it is filled with a blood clot the surface of which is covered by debris. The clot consists of a network of fibrin with an uneven distribution of blood cells. A dense aggregation of erythrocytes may be seen next to the former periodontal membrane. The socket from the corresponding animal treated with sulphonamide cone is shown in Fig. 2. Apparently the cone has only been pushed partly down into the socket, of which the upper part is occupied by a mass of erythrocytes around numerous sulphonamide particles.

Four days after the extraction the organization of the clot is well advanced with active ingrowth of capillaries and fibroblasts (Fig. 3). Resorption of the alveolar bone is pronounced. In the treated socket (Fig. 4) sulphonamide particles may be seen at the bottom and along the lingual border of the socket, in which the clot is undergoing organization in a degree comparable to the control animal. Examined under higher magnification (Fig. 5) this area reveals small sulphonamide particles intimately surrounded by young connective tissue. There is no indication of inflammation or foreign body reaction (multinucleated giant cells).

The formation of new bone is initiated about 4 days after the extraction in both treated and control animals. On the seventh day it is more advanced, and very active bone formation may be seen at the bottom of a socket from a treated animal (Fig. 6).

It is noted how the new bone forms in the young connective tissue in close proximity to numerous sulphonamide particles. After 14 days bony healing is almost complete. Also at this stage treated and untreated animals show no difference in the amount of bone formed. Fig. 7 illustrates the socket from a treated animal. Extensive new bone formation is seen, and in the central part of the socket, sulphonamide particles may be seen lying in fibrous tissue. A higher magnification of this area (Fig. 8) shows how new bone is formed directly upon these particles.

Forty-nine days after the extraction the sockets of both control animals and treated animals are filled with bone (Figs. 9 and 10). A small abscess is seen in the sub-epithelial tissue of the control animal, but the bone structure of these two socket areas is almost identical. When the marrow cavities of the treated animal are examined under higher magnification (Fig. 11) the presence of sulphonamide particles is revealed. These particles are lying in the marrow tissue without any indication of inflammatory or foreign body reactions.

Epithelial healing appeared identical in treated and untreated animals.

Whenever particles were found in the healing sockets of the treated animals, they were examined in polarized light. In most instances they appeared to be birefringent. Fig. 12 illustrates this condition in an animal 14 days after the extraction: all the bright circular or semicircular areas represent the particles, whereas the more diffuse birefringent light stems from collagen.

DISCUSSION

It can reasonably be assumed that the particles described in the sockets of the treated animals really are particles of sulphonamides. The majority of these particles were birefringent when viewed in polarized light and, furthermore, when sections of the cones were examined with transmitted ordinary and polarized light the appearance of the particles was the same as those in the tissues. That not all particles show birefringence may be due to a washing out of the crystalline material. Whether this happens while the particles are in the rat mandible or during the histologic preparation, or both, cannot be decided from this study, but the latter seems most likely. That sulphonamide particles may remain in human tissues for a considerable period was shown by GUSTAFSON (1958), who demonstrated the particles as late as 10 years after the treatment. In our rat material the particles were present 49 days after the extraction, the longest post-operative period examined. There was no striking diminution in the number of particles in the animals of this group as compared to the post-operative periods of a few days.

No demonstrable inhibition of fibroblast proliferation by the sulphonamide particles was noted in this study. This is not in agreement with the results of GLYNN (1941). He used a refined technique, whereby he was able to apply the principle of cell counting for the estimation of fibroblastic inhibition. By this method only a slight, barely significant, inhibition could be demonstrated from the use of sulphathiazole, but not from the use of sulphanilamide. In the light of this, it is not to be expected that any inhibitory effect of the mixture of sulphanilamide and sulphathiazole

used in this study could be detected by the relatively crude method applied. No foreign body reaction was seen, nor were there any signs of inflammatory reaction. That GUSTAFSON (1958) found both of these tissue reactions in a human subject may be explained by the extremely long post-operative period and by the primary infectious nature of the condition. It is known that the rat is highly resistant to infection, but also that foreign body reactions may occur (GILBERTSON and CLARK, 1958).

With regard to bone formation, no inhibition of the initial bone formation was noted. As healing progressed, it was seen that the osteoblasts were able to form bone even in intimate contact with the sulphonamide particles (Fig. 8). Large masses of sulphonamide particles would, of course, constitute a physical hindrance for bone formation, but in the present study it was considered of minor importance as far as final bony healing was concerned.

Acknowledgement—This work was supported by a grant from the Danish Dental Association.

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PLATE I

FIG. 1. Control rat, 24 hr after extraction, normal blood clot. Haematoxylin and eosin. $\times 27$.

FIG. 2. Treated rat, 24 hr after extraction, showing sulphonamide particles surrounded by erythrocytes in upper part of clot. Haematoxylin and eosin. $\times 27$.

FIG. 3. Control rat, 4 days after extraction, showing ingrowth into the clot of young fibrous tissue. Haematoxylin and eosin. $\times 27$.

FIG. 4. Treated rat, 4 days after extraction, showing sulphonamide particles in the organizing clot. Haematoxylin and eosin. $\times 27$.

THE INFLUENCE OF SULPHONAMIDE CONES UPON SOCKET HEALING IN THE RAT

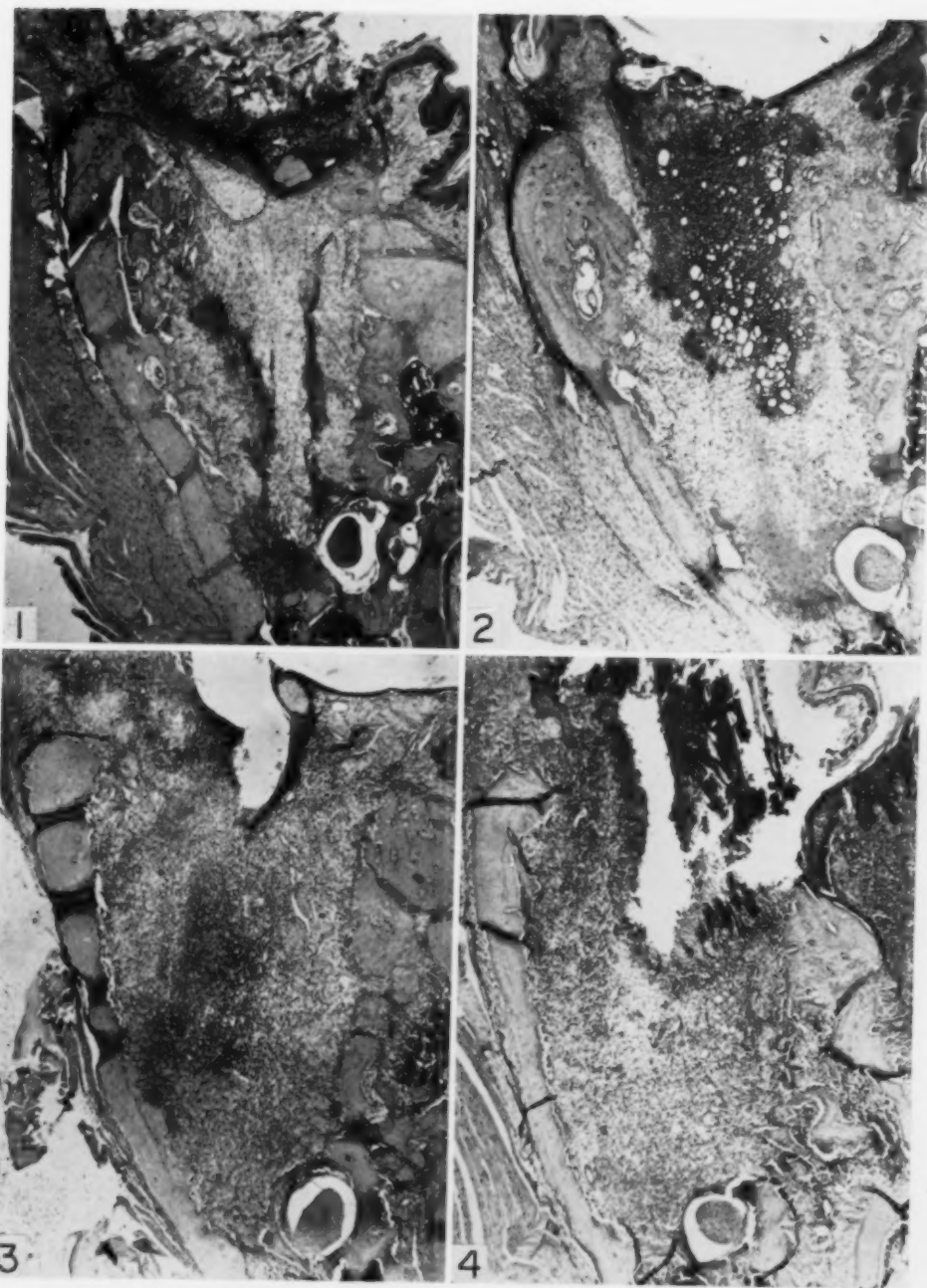


PLATE I

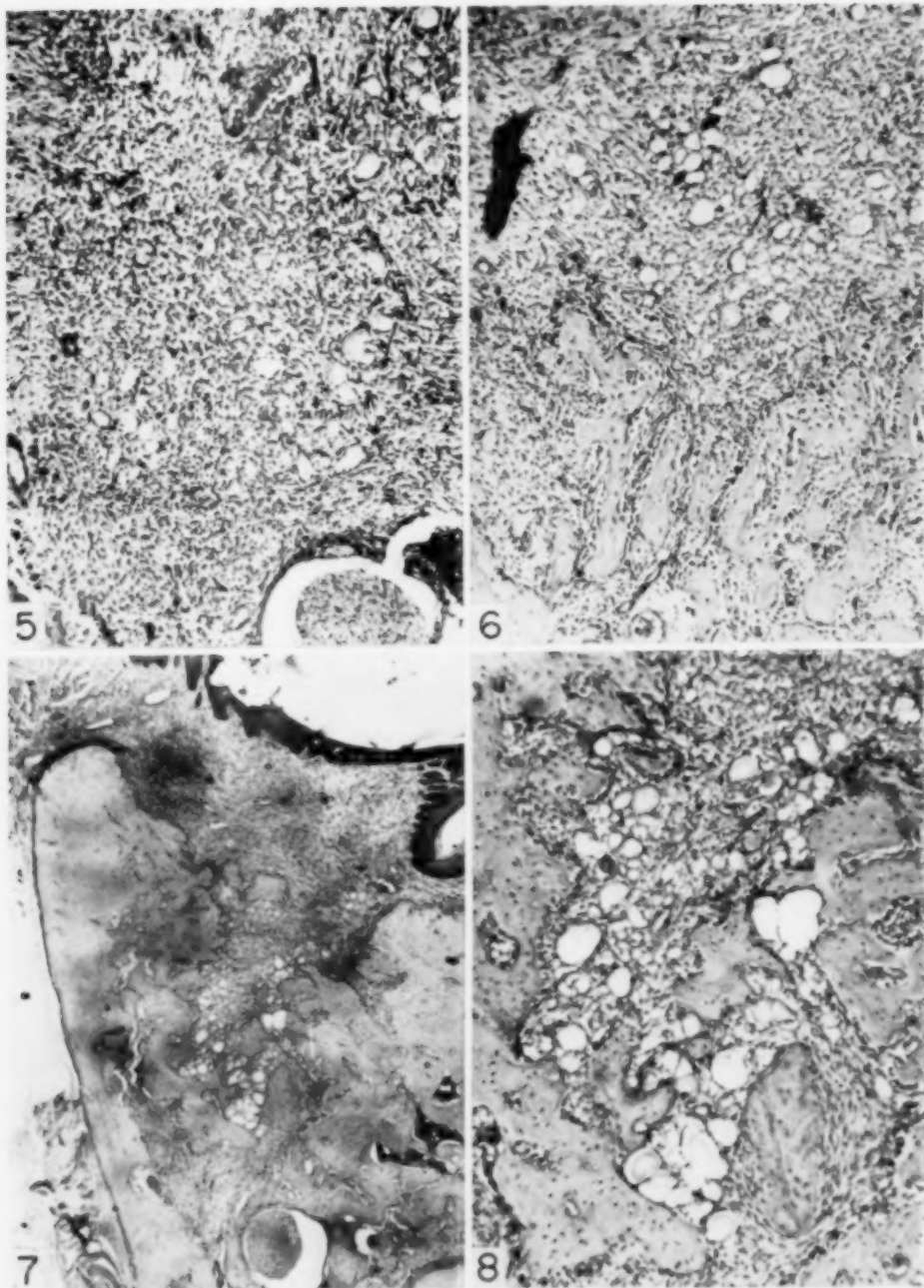


PLATE 2

PLATE 2

FIG. 5. Higher magnification of the fundus area from Fig. 4, sulphonamide particles in young fibrous tissue. Haematoxylin and eosin. $\times 72$.

FIG. 6. Socket of treated rat, 7 days after extraction. Detail from the fundus area showing formation of new bone in close proximity to sulphonamide particles. Haematoxylin and eosin. $\times 72$.

FIG. 7. Socket of treated rat, 14 days after extraction. Extensive formation of new bone with sulphonamide particles in centre of socket. Haematoxylin and eosin. $\times 27$.

FIG. 8. Higher magnification of particles from Fig. 7, new bone bordering upon particles. $\times 75$.

PLATE 3

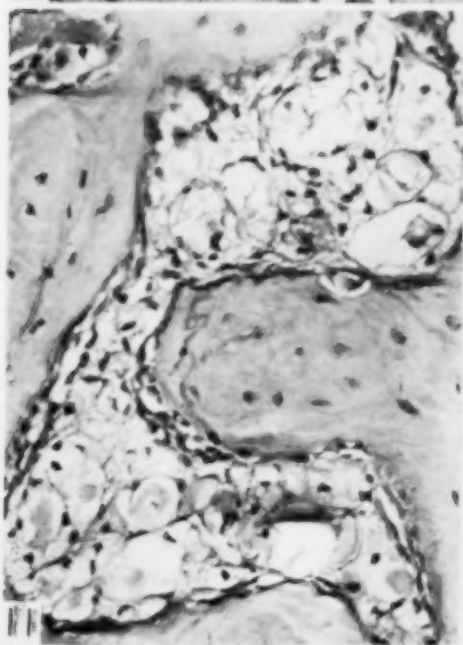
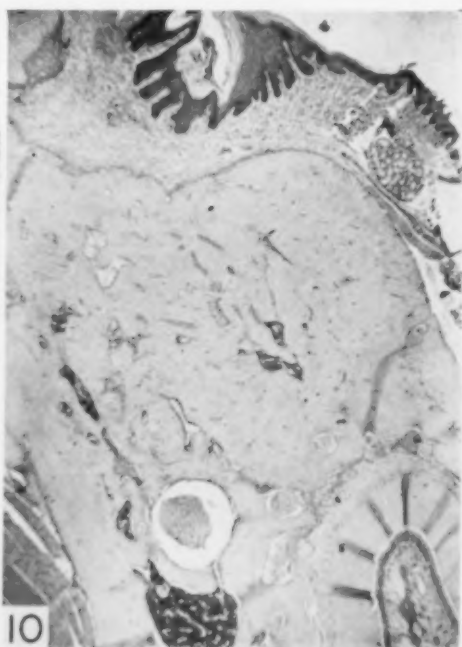
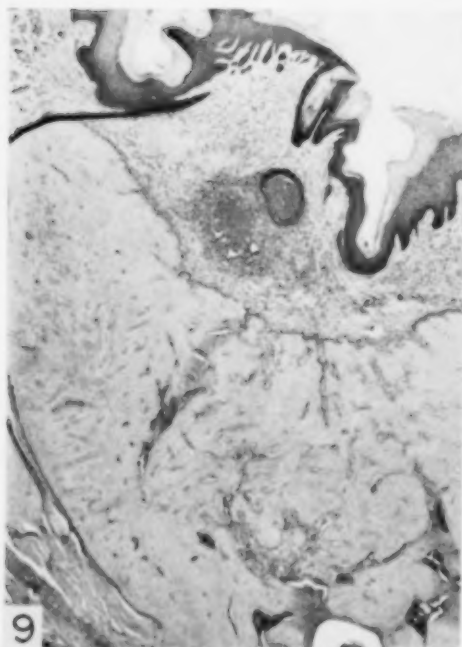
FIG. 9. Control rat, 49 days after extraction. Socket area filled with bone. Small abscess in the subepithelial tissue. Haematoxylin and eosin. $\times 27$.

FIG. 10. Treated rat, 49 days after extraction. Socket area filled with bone. Haematoxylin and eosin. $\times 27$.

FIG. 11. Higher magnification of marrow space from upper, buccal part of socket from Fig. 10. Sulphonamide particles situated in the marrow tissue. $\times 288$.

FIG. 12. Detail of socket from treated rat 14 days after extraction, viewed in polarized light. Distinct birefringence of sulphonamide particles, more diffuse birefringence related to collagen. $\times 70$.

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Vol.

5

1961

SENSORY NERVE TERMINATIONS IN THE ORAL MUCOSA

A. D. DIXON

Department of Anatomy, University of Manchester, England

Abstract—Methylene blue and silver impregnation techniques were used to study the morphological features of sensory nerve terminations in selected regions of the oral mucosa from human subjects and a number of animals. Sensory nerve endings vary widely in their morphology and consist of free or organized terminations. Organized terminations can be classified as being of a simple, complex or compound coiled type. Nerve terminations differ sufficiently in histological detail to prevent their accurate correlation with specific sensations. Consequently the theory of sensibility which is favoured suggests that sensation is a function of the pattern of responses of nerve fibres reaching higher centres.

INTRODUCTION

THE SENSATIONS which may be experienced in the oral cavity through the medium of sensory nerves in the oral mucosa are similar to those which follow skin stimulation. It has been suggested frequently that within the skin each modality of sensation is associated in some way with an end-organ or nervous pathway which is specific, in that it will only conduct to consciousness its own peculiar sensory quality (HAM, 1957; BAILEY, 1958; BUCHANAN, 1961). This theory of the specificity of nerve terminations is based on the findings of WAGNER and MEISSNER (1852), KRAUSE (1860), VON FREY (1894) and WOOLLARD (1935). In complete contrast is the view which maintains that the ability to distinguish one type of sensation from another is a mere reflection of some characteristic difference in the pattern of nervous impulses reaching higher centres in the nervous system from non-specific peripheral end organs (NAFE, 1929; BISHOP, 1946; HAGEN *et al.*, 1953; WEDDELL, PALMER and PALLIE, 1955).

Reference to the presence of nerve endings in the sub-epithelial tissue of the oral mucosa can be found in the older anatomical texts (QUAIN, 1891). BOTEZAT (1907) and CECCHERELLI (1908) were amongst the first to make specific studies of oral nerve terminations and the latter author illustrated his findings by numerous drawings, which showed a great variety of endings comparable to those found in the skin. Many investigations of oral sensory mechanisms have been concerned with specific regions, notably the gingiva (JURIEWA, 1913; KADANOFF, 1928; LEWINSKY and STEWART, 1938; BRADLAW, 1939; RADDEN, 1945). GAIRNS (1956) compared nerve endings in the gingiva with those in the tongue and palate and found them to be morphologically similar in all three situations.

In view of the multiplicity and degree of complexity of cutaneous and oral sensory nerve terminations which have been described in the literature it was considered important to re-examine the morphological characteristics of these structures in

various parts of the mouth, before proceeding to the study of other aspects of oral sensation (DIXON, 1957). Not only would the results of such an investigation enable a reassessment of the findings of other workers to be made, but they could throw further light on our understanding of oral sensation. In particular a morphological classification of oral nerve terminations might be established and evidence provided for the concept of either a specific or a patterned theory of oral sensibility.

MATERIALS AND METHODS

The investigation was based on a neurohistological study of selected parts of the oral mucosa from forty-five normal animals, including the monkey, cat, rabbit and guinea-pig. Modifications of supravital or intravital methylene blue techniques (MITCHELL, 1953) and a Bielschowsky-Gros silver impregnation method (GARVEN and GAIRNS, 1952) were employed. In some animals in which methylene blue was the stain of choice 0.5 ml of a solution of hyaluronidase (1 mg/5 ml normal saline) was infiltrated into the selected site and gently massaged into the tissues. After 20 min 1-2 ml of a 0.01-0.25% solution of methylene blue was injected into the same area and permitted to stain the tissues for a further 10-20 min. Staining was completed supravitaly and whole thickness preparations or frozen sections (40-50 μ) were prepared for study. This procedure resulted in a greater uniformity of nerve fibre staining and the prevention of staining artifacts, as had been the experience of other investigators in different tissues (WEDDELL and PALLIE, 1954).

The silver diamine method (GARVEN and GAIRNS, 1952) gave the most consistent results. Two modifications of their method which had a marked effect on the quality of the preparations were significant. Firstly, frozen sections of the mucosa were passed through three changes of 20% formaldehyde in tap water prior to immersion in the reducing bath. Neutralized formalin, as recommended by GARVEN and GAIRNS (1952) for this step, had a deleterious effect in that heavy precipitates were formed at the subsequent reduction stage. Secondly, de-ionized water was used in place of glass-distilled water throughout the procedure, and as a result the tendency towards precipitate formation was further reduced. Both modifications appeared to be associated with the nature of the water supply in the laboratory, although subsequently they have been applied elsewhere with equal success.

Essentially a comparative study, the findings in animals were correlated with those in man by the examination of silver preparations of tissue from eighteen human subjects, removed either at surgery or soon after death. The age range of this material was from 16 to 65 years. In both animal and human material the gingiva, lips, cheeks, tongue, hard and soft palates were the regions from which tissue was selected for investigation. Several routine histological methods for non-nervous tissues were used as controls for the neurohistological techniques.

RESULTS

The finer structure of the sub-epithelial collagenous tissue of the oral mucosa can be demonstrated by silver methods for reticulin and these techniques frequently revealed the delicate adventitial capsules of nerve terminations, which occupied a

large number of the sub-epithelial papillae (Fig. 1). Many of the capsules were closely related to the basement membrane of the epithelium and were transversely striated due to the presence of encircling reticular fibres and the processes of small fibrocytes. Fig. 2 shows a coiled nerve ending from a similar situation in which the nerve fibre content has been selectively stained. The stem axon and its terminal ramifications are seen clearly but the capsular material is less distinct.

The wealth of sensory terminations which had been observed in the oral mucosa was not readily subdivided into distinct morphological groups. They consisted of fine non-myelinated terminal filaments which often ended freely in the epithelium, and of many organized terminations of varying degrees of complexity. The term "organized termination" was used to define any closely woven or coiled nerve terminal, whether or not it possessed a distinct tissue capsule. These terminations were located mainly in the summits of the sub-epithelial papillae and involved all gradations, from simple loops to highly complex coiled endings.

Free nerve terminations

In Fig. 3 a sub-epithelial nerve plexus gives origin to numerous freely ending nerve fibres which enter every sub-epithelial papilla. Careful examination showed that many of the fibres did not lose their myelin sheaths until they approached close to the epithelium where they commonly divided into two or more terminal branches. These branches passed either close to the basal layers of the epithelium or between the basal cells to form extremely fine intra-epithelial fibres.

Free nerve endings have been seen in all parts of the oral mucosa and in all the animals studied. Nerve terminations of this type which were stained by the silver impregnation method are shown in Fig. 4. Two small parallel bundles of nerve fibres pass towards the epithelium and in one bundle the fine terminal non-myelinated fibres are in sharp focus for a considerable part of their course. Those indicated by an arrow could be traced between the basal layers of the epithelium. The fibres in the adjacent bundle are seen distinctly only for a short part of their length for they pass into a deeper plane of section.

The elongated form of the sub-epithelial papillae (Fig. 5) is characteristic of human gingival tissue. In one papilla a group of four or five nerve fibres ascends to end just below the basement membrane. One fibre terminates as a small expansion or neurofibrillar enlargement. Other fibres, which occupy a deeper plane, are much finer and fainter in appearance. When examined under the microscope free endings of this type often showed an increasingly pronounced neurofibrillar structure and granularity in their terminal portions, which made it difficult to determine their exact point of termination. In contrast the other papilla in Fig. 5 shows a small blood vessel, with distinct nuclei of endothelial cells, which might be mistaken on casual observation for a group of free nerve endings.

The formation of intra-epithelial nerve fibres is shown on higher magnification in Fig. 6. Thickly myelinated fibres are a prominent feature and thin naked filaments, which could be followed for a short distance into the epithelium are indicated by arrows. Part of a nerve bundle which belongs to the sub-epithelial nerve plexus, and

consists of a number of myelinated nerve fibres of various diameters, lies towards the lower margin of the photograph. A thick nerve fibre gives origin to a coiled nerve termination which is superimposed upon the basal epithelial cells close to the intra-epithelial fibres.

Free nerve terminations were observed not only as relatively straight fibres, which ended within the epithelium as inter-cellular filaments, but also in the form of simple loops which on occasions re-entered the sub-epithelial connective tissue. In some instances, as in human gingiva, loops of this type were found in close proximity to the surface layers of the epithelium.

Organized terminations

Organized nerve endings varied considerably in size and structure but appeared to have the same essential characteristics in all parts of the oral mucosa. They are classified as being of a simple, complex or compound variety.

Simple coiled terminations. A simple, loosely wound coiled receptor is shown in Fig. 7. The nerve fibres which take part in its formation are of variable thickness and are noticeably varicose. Small swellings midway along the left margin of the termination may be end-bulbs, but are more likely to be due to incomplete staining, or to the appearance of the cut ends of severed nerve fibres. A silver preparation of human palatal mucosa (Fig. 8) demonstrates similar loosely coiled terminations. One coil occupies an area of connective tissue which is the summit of a sub-epithelial papilla. The coil is composed of at least two myelinated nerve fibres and gives off branches which pass upwards into close relationship with the epithelium. Near the upper margin of the figure the branches form intra-epithelial nerve fibres. Towards the left two more nerve fibres sweep round the periphery of another papilla and form part of a second coil.

A more tightly coiled termination (Fig. 9) is associated with stem fibres which ascend and are at first indistinct, where they are deeply placed. Two of the stem fibres turn obliquely towards the left, ending abruptly after a short distance where they have been cut by the microtome knife. The remaining fibre continues upwards and divides into two branches which form the coiled ending. A short distance below the coil two fibres are apparent, which suggests that this termination is a "closed" coil, formed by a single fibre which ascends from deeper tissues, turns upon itself several times and retraces its course.

Coiled terminations of similar configuration are presented in Figs. 6, 10, 11, although more than one fibre is concerned in the formation of some of the receptors. The nerve coil (Fig. 10) is characterized by prominent "terminal" swellings and localized thickenings of the constituent fibres. The stem fibres are myelinated and Schwann cell nuclei can be seen. In Fig. 11 the thick stem axons, which give rise to the compact ending, can be traced readily into continuity with the sub-epithelial nerve plexus and localized thickenings of the nerve fibres within the coil are evident. The neurofibrillar structure of the thickenings could be demonstrated, especially when the degree of silver staining was not excessive (Fig. 12).

Simple coiled terminations were of common occurrence also in methylene blue preparations (Fig. 13). This coil is very similar to that shown in Fig. 10 and at least three fine beaded nerve fibres take part in its formation. Elsewhere beaded fibres lie adjacent to the basement membrane, or form loops, and they should be compared with the fine fibres, probably of autonomic origin, which can be seen in Fig. 11.

Complex coiled terminations. A receptor of a more complex type is shown in Fig. 14. The varicose nature of several of the stem fibres will be noted. A small number of accessory fibres which are extremely fine and possess much smaller beads along their length are visible. Occasionally these finer fibres by-pass the coils to form delicate sub-epithelial nets or intra-epithelial filaments. Fig. 15 shows a more unusual form of complex nerve ending consisting of two simply coiled parts intimately interconnected by several nerve fibres. Only the lowermost of the coiled parts is seen in this photograph, for the other is masked by the basal layers of the epithelium. The termination is supplied by fibres which ascend to both coiled portions.

Compound coiled terminations. Occasionally the arrangement just described is carried to an extreme and large terminations consisting of a number of closely associated coiled endings is the result. Individually these coils may be of extremely simple form (Fig. 16) whilst in other areas they have been found to incorporate more complexly coiled terminations. Many of the coils are incomplete for, due to the manner in which they are clustered around the stem axons in grape-like fashion, inevitably some have been sectioned. The cut ends of nerve fibres are readily recognized, in contrast to the greater difficulty experienced in determining the nature of some of the terminal swellings in Fig. 10. Many of the axons in this compound endings are myelinated and possess clearly defined Schwann cell nuclei. In some instances the myelination of the fibres may be followed almost to the commencement of coiling of their terminal parts.

DISCUSSION

The outstanding feature of the nerve terminations in the oral mucosa is the great variation in their morphology, an observation made by many previous workers including CECCHERELLI (1908), JURIEWA (1915), KADANOFF (1928), LEWINSKY and STEWART (1938), BRADLAW (1939) and GAIRNS and AITCHISON (1950). The existence of many morphological types of endings has led in the past to attempts to classify them, either on the basis of their histological characteristics (KOKUBUN, 1929) or their positions relative to the epithelial or sub-epithelial tissues (GAIRNS, 1955). Classifications also vary considerably in their complexity and CECCHERELLI (1908), believing that no simple subdivision was possible, described many groups of nerve terminations. BRADLAW (1939) and RADDEN (1945) advocated much simpler classifications.

While agreeing with HAGEN *et al.* (1953) and WEDDELL *et al.* (1955) that the diversity in the form of nerve endings in mucous membrane precludes any rigid classification, it seems desirable to adopt some scheme of grouping which will indicate the major types observed in the oral mucosa. The subdivisions of free, simple, complex and compound coiled nerve endings are judged as being suitable for this purpose.

Such a classification can be applied readily, not only to endings found in various parts of the mouth but also, from the comparative anatomical standpoint, to those observed in the mucosa of lower animals. Often in these animals the nerve endings are of a simple type and are less numerous, confirming the findings of ABE (1954) and KAMADA (1955). In addition, this method of description does not imply any unwarranted relationship between the type of nerve ending and the modality, or modalities of sensation which it subserves.

The correlation of the individual types of nerve terminations in mucous membrane and skin, with the functions which they fulfil in peripheral sensation, has been the object of much criticism in recent years (HARPMAN, 1951; SINCLAIR, WEDDELL and ZANDER, 1952; SINCLAIR, 1955). HARPMAN (1951) observed that the mucous membrane over the nasal conchae is sensitive to ill-defined pain, cold, warmth and touch although only free nerve endings could be demonstrated. SINCLAIR *et al.* (1952) found in the skin of the human external ear that there are no organized nerve endings, only free nerve terminals or basket-like networks around the roots of hairs. Nevertheless cutaneous sensibility is adequately represented in all its modalities. SINCLAIR (1955) suggested that the differences between nerve endings are too subtle for present histological methods to detect, and their variety too great, to allow a one to one relationship between sensory endings and a tetrad of modalities.

The results of the present investigation provide confirmatory evidence in the oral mucosa. Although nerve endings in oral tissues can be classified on a simple basis it will be apparent from the limited selection which has been shown that there are many variations. These differ sufficiently in histological detail to prevent their correlation with specific sensations. Indeed it appears quite possible that, as in the skin, many of the nerve endings may subserve more than a single sensory function. Consequently the "patterned" rather than the "specific" theory of sensibility is favoured. The nature of sensation which may be evoked from the oral mucosa may be dependent not only upon the manner of stimulation of a group of nerve terminations, but also upon the pattern of responses within the nerve fibres reaching higher nervous centres. However until the physiological behaviour of the various endings under discussion is more fully understood one can only speculate on this aspect of the problem.

Acknowledgements—I am indebted to Professor G. A. G. MITCHELL for his helpful criticism throughout the period of research and to Mr. F. T. MONKS of the Turner Dental School, University of Manchester, who provided much of the human material. Technical assistance given by Mr. C. K. PEARSON is gratefully acknowledged and it is a pleasure to record the skill of Mr. P. HOWARTH who was responsible for the photomicrography.

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PLATE I

FIG. 1. Adventitial capsule (A) of a sensory nerve termination lying in contact with the basement membrane of the stratified epithelium in the hard palate of a monkey. Wilder's method for reticulin. $\times 380$.

FIG. 2. A coiled nerve ending (C) in the sub-epithelial connective tissue in the hard palate of a cat. S=stem axon. Silver impregnation method. $\times 570$.

FIG. 3. A sub-epithelial nerve plexus (P) composed of thick bundles of myelinated nerve fibres gives rise to free terminations (F) which occupy sub-epithelial papillae. The darkly stained nuclei of other cellular elements are scattered throughout this section from the dorsum of the tongue of a rabbit. Methylene blue. $\times 95$.

FIG. 4. Free nerve endings (F) in a sub-epithelial papilla in the lip of a monkey. The arrow indicates fine nerve fibres which could be traced into the epithelium in the original specimen. P=pigment cell. Silver impregnation method. $\times 290$.

FIG. 5. Free nerve endings (F) in a human gingival papilla. SP=sub-epithelial papilla. E=neurofibrillar enlargement. V=small blood vessel. Silver impregnation method. $\times 280$.

FIG. 6. Intra-epithelial nerve fibres (arrowed) in the dorsum of the tongue of a monkey. C=coiled nerve termination. M=myelinated nerve fibre. P=part of the sub-epithelial nerve plexus. Silver impregnation method. $\times 380$.

SENSORY NERVE TERMINATIONS IN THE ORAL MUCOSA

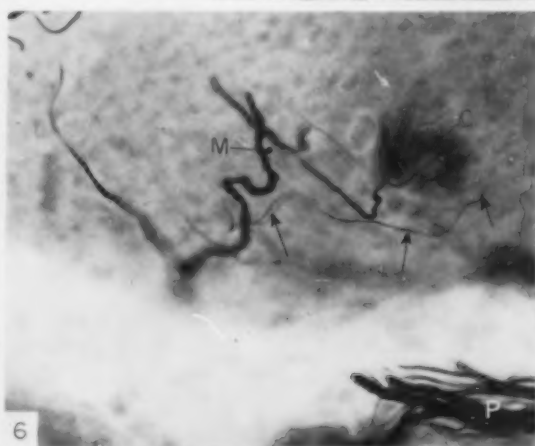
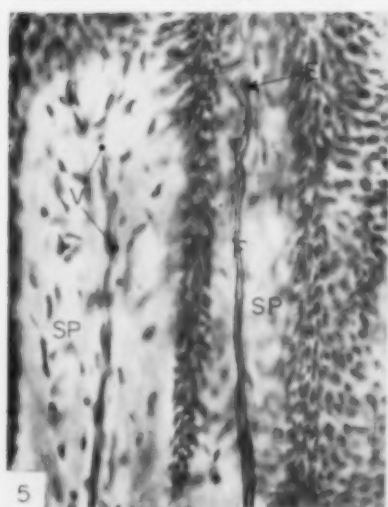
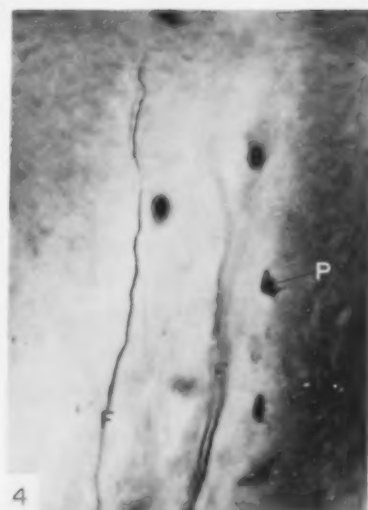
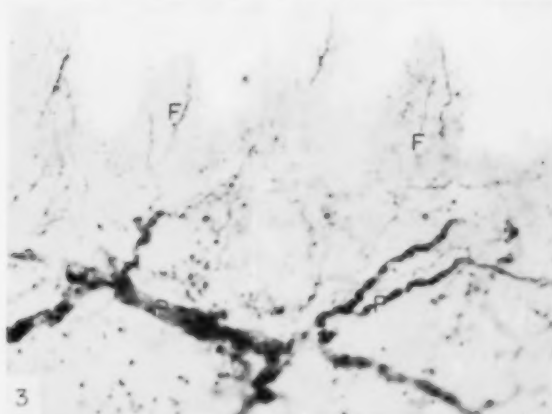
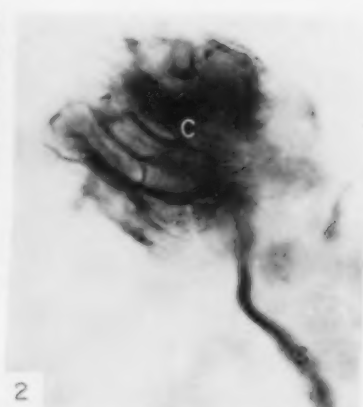
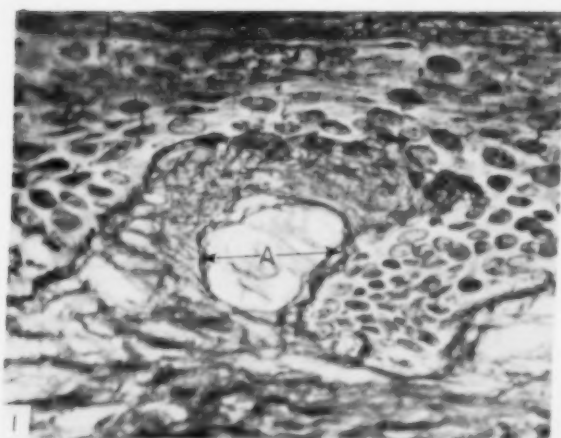
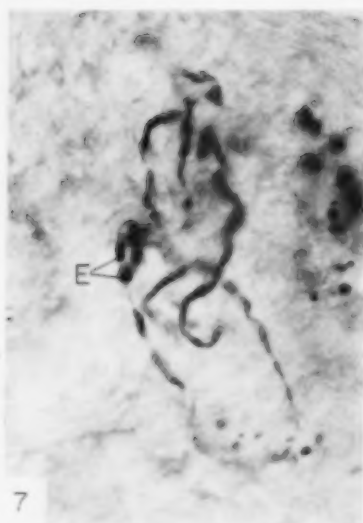
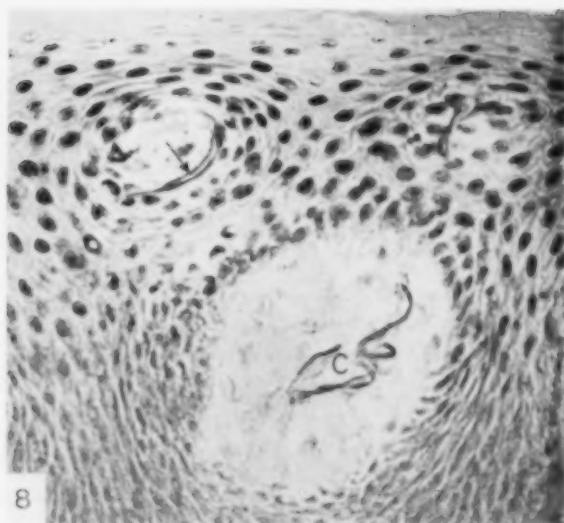


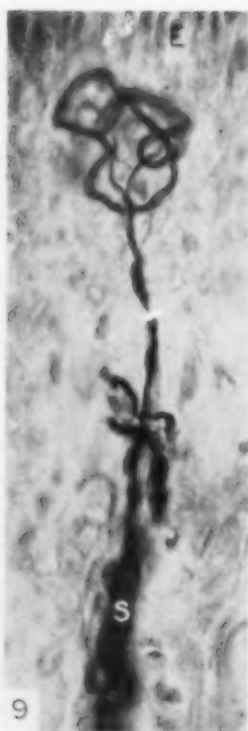
PLATE I



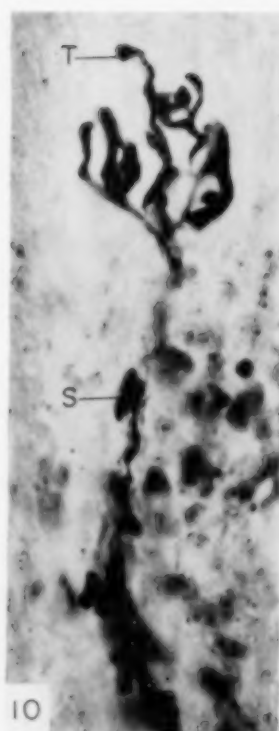
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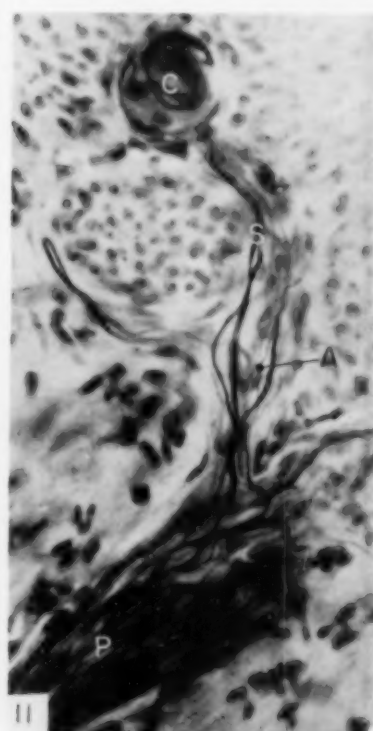
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PLATE 2

FIG. 7. Simple coiled nerve ending in the dorsum of the tongue of a guinea-pig. E="end-bulbs" which are probably an artifact appearance. Methylene blue. $\times 450$.

FIG. 8. A simple coiled nerve ending (C) in human hard palate. Part of a second simple coiled formation (arrowed) can be seen in the upper left corner of the photomicrograph. Silver impregnation method. $\times 430$.

FIG. 9. Coiled nerve ending in human hard palate. S=stem axons. E=epithelial cells. Silver impregnation method. $\times 430$.

FIG. 10. Coiled nerve ending in the dorsum of the tongue of a monkey. S=nucleus of a Schwann cell. T="terminal swelling". Silver impregnation method. $\times 430$.

FIG. 11. Tightly coiled nerve ending (C) in the hard palate of a cat showing the stem fibres (S) arising from the sub-epithelial nerve plexus (P). Note the associated fine fibres (A) which may be of autonomic origin. Silver impregnation method. $\times 320$.

PLATE 3

FIG. 12. Transverse section through a coiled nerve termination in the hard palate of a cat. NF=neurofibrils. A=outline of advential capsule. Silver impregnation method. $\times 910$.

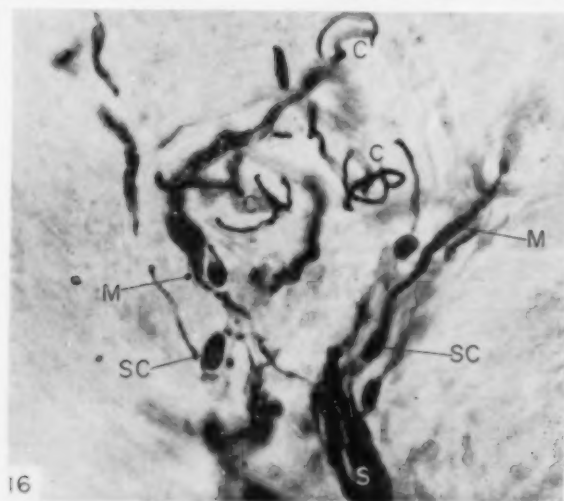
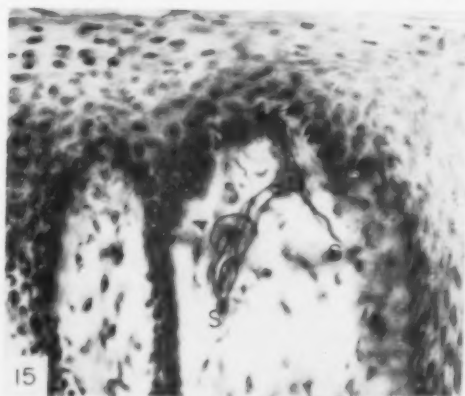
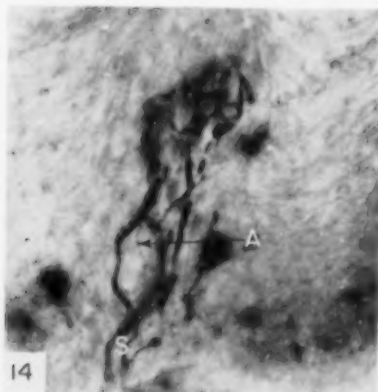
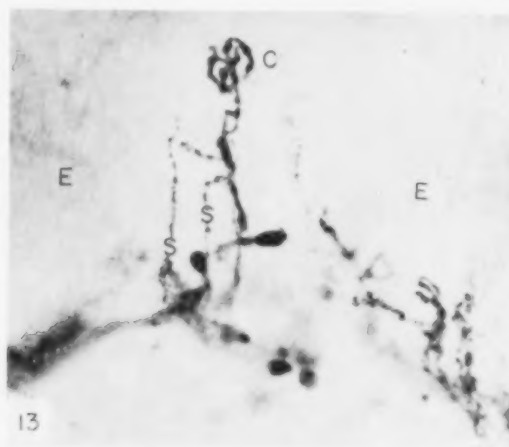
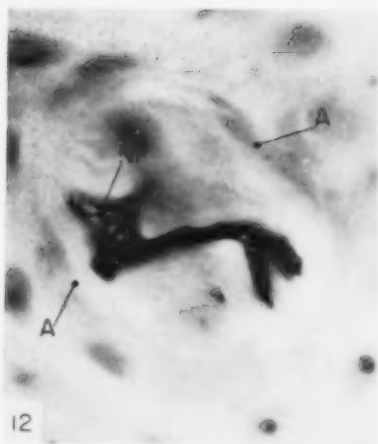
FIG. 13. A simple coiled nerve termination (C) in the lip of a monkey. Note the beaded nature of associated nerve fibres (S) and those which lie along the basement membrane zone. Some of the gross beading may be a staining artifact. E=epithelium. Methylene blue. $\times 315$.

FIG. 14. A complex coiled nerve ending in the dorsum of the tongue of a guinea-pig. S=stem axons. A=accessory beaded nerve fibre. Methylene blue. $\times 385$.

FIG. 15. A complex coiled nerve termination in a human gingival papilla. Part of the complex (arrowed) is masked by the basal layers of the epithelium. S=stem axons. Silver impregnation method. $\times 290$.

FIG. 16. Compound coiled nerve ending in a sub-epithelial papilla in the hard palate of a monkey. S=stem axon. SC=nucleus of a Schwann cell. M=myelin sheath. C=simple nerve coil. Silver impregnation method. $\times 380$.

SENSORY NERVE TERMINATIONS IN THE ORAL MUCOSA



Vol.
5
1961

A QUANTITATIVE COMPARISON OF THE AMINO ACID COMPOSITION OF SOUND DENTINE, CARIOUS DENTINE AND THE COLLAGENASE RESISTANT FRACTION OF CARIOUS DENTINE

W. G. ARMSTRONG

Institute of Dental Surgery (University of London),
Eastman Dental Hospital, London W.C.1, England

Abstract—Using ion-exchange chromatography a quantitative comparison was made of the amino acid composition of the organic matrices of human sound dentine (SD) and carious dentine (CD) preparations. The amino acid content of CD was essentially collagenous in character, though compared with SD increased concentrations of the following amino acids were observed: aspartic, threonine, serine, glutamic, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Marked reductions of arginine (27 per cent loss), proline (30 per cent loss) and hydroxyproline (22 per cent loss) were also observed in CD hydrolysates. A fraction of the CD matrix, totally resistant to collagenase disintegration (CD/R Fraction), contained 13.6 per cent carbohydrate: the protein component was similar to CD in amino acid composition, but with a further marked decrease in arginine content (67 per cent loss). Various possible interpretations of the results are discussed and it is considered that they indicate intrinsic modifications during caries involving the arginine, proline and hydroxyproline components of the matrix, together with some degree of proteinaceous contamination and proteolytic degradation. The results are also examined in the light of previous work on possible factors determining collagenase resistance of the dentine matrix.

INTRODUCTION

EARLIER investigations showed that modification of the dentine occurred during caries, converting the organic matrix to a form which was partially resistant to attack by the enzyme collagenase (ARMSTRONG, 1958a). It was also demonstrated that artificial modification or destruction of the amino groups, guanidyl groups and hydroxy groups in the organic matrix of sound dentine would convert it to collagenase-resistant forms (ARMSTRONG, 1958b). It was further observed that in comparison with sound dentine the matrix of carious dentine contained significant amounts (ca. 4 per cent) of carbohydrate material, and that a fraction of carious dentine completely resistant to collagenase attack contained approximately 11 per cent carbohydrate (ARMSTRONG, 1960).

It was considered that the particular properties associated with carious dentine might be explained in terms of modifications to the collagenous matrix, involving destruction, removal or masking of certain amino acid units. Modifications to the dentine matrix during caries, which involved the amino acid components, might be

reflected in altered concentrations of certain amino acids in carious dentine hydrolysates. Thus any modification involving destruction of specific amino acid side-chains of the matrix collagen would be reflected as reduced concentrations of these amino acids in hydrolysates of the carious dentine. Similarly, modifications involving masking reaction of side-chain groupings might also be reflected in altered amino acid concentrations in hydrolysates, provided that during the hydrolytic process a simple reversal of any such modification did not occur. Specific removal of peptides rich in certain amino acids would also be reflected in changed concentrations of these amino acids in hydrolysates of the residual matrix.

It was, therefore, considered that a comparison of the amino acid composition of sound dentine with carious dentine might provide indications of changes which had occurred to the dentine matrix during the carious process.

MATERIALS AND METHODS

Preparation of dentine samples

(i) *Sound and carious dentine.* Sound and carious dentine was collected, prepared and demineralized with EDTA as previously described (ARMSTRONG, 1958a). Incineration gave ash values of approximately 1 per cent.

(ii) *Preparation of carious dentine fraction completely resistant to collagenase attack.* Approximately 200 mg of dried demineralized carious dentine powder was weighed into a 6×1 in. Pyrex tube. 20 ml of a 50 mg per cent solution of collagenase in M/15 phosphate buffer, pH 7.3, was added and the tube stoppered. The collagenase

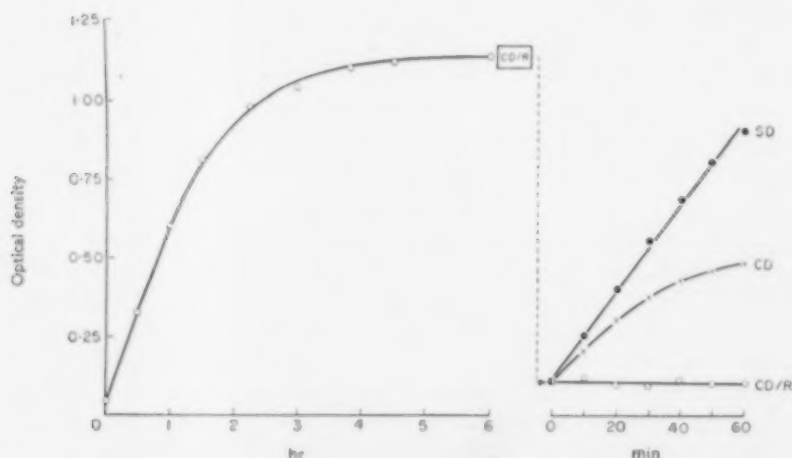


FIG. 1. Preparation of CD/R fraction. Left hand curve shows optical density values for α -NH₂ N liberated by collagenase action on CD. After 6 hr incubation at 37°C the residual CD/R was collected and treated as described in text. On treatment with fresh collagenase solution (right hand curve) it was shown to be totally resistant to attack, compared with sound dentine (SD) and the partially resistant carious dentine (CD).

preparation used was a freeze-dried preparation (B 637 C) kindly donated by the manufacturers (The Wellcome Research Laboratories). Data on the preparation are given in an earlier publication (ARMSTRONG, 1958a). The collagenase/carious dentine suspension was then incubated, with shaking, at 37°C in a thermostatically controlled water bath. 0.2 ml aliquots were removed at suitable intervals for α -NH₂-N assay by the ninhydrin method. The rate of α -NH₂-N release slowly decreased and eventually stopped after approximately 6 hr incubation period (Fig. 1). The residual carious dentine particles were collected by centrifugation, washed several times with water, then with acetone and air dried. This carious dentine preparation is referred to as the CD/R fraction. Its resistance to further collagenase disintegration was tested against a freshly prepared collagenase solution, when it was shown to be totally resistant to disintegration by the enzyme (Fig. 1).

Preparation of hydrolysates

Approximately 10–15 mg quantities of each preparation were accurately weighed into Pyrex tubes, 5 ml of 6 N HCl added, the tubes sealed and immersed in a refluxing water bath (100°C) for 24 hr. The tubes were opened and the contents quantitatively transferred to evaporating dishes. The hydrolysates were then evaporated to dryness, *in vacuo*, over P₂O₅ and NaOH. Each residue was dissolved and made up to a measured volume with distilled water, and a suitable aliquot (usually 2 ml) applied to the column.

Ion-exchange chromatographic separation of amino-acids

The procedure was fundamentally similar to the original MOORE and STEIN (1951) technique as modified by EASTOE (1955) for the analysis of collagen hydrolysates. Dowex 50 × 12 resin, passing 200 mesh screens, was used in 150 × 0.9 cm and 15 × 0.9 cm jacketed chromatography columns. A Locarte Automatic Fraction Collector, Model LDC/2, was used for the collection of the 1 ml effluent fractions, which were analysed by the modified ninhydrin method described by JACOBS (1956) using 1 ml of reagent per fraction.

Hydroxyproline and proline

Because of the overlap of the hydroxyproline and aspartic acid peaks estimation of hydroxyproline was made independently using MARTIN and AXELROD's (1953) modification of NEUMAN and LOGAN's (1950) method, as described by BLOCK and WEISS (1956). Total hydroxyproline-plus-proline estimations were made using the TROLL method (1953), also described by BLOCK and WEISS. Calculation of the proline content, by difference, gave more reliable results than the ninhydrin estimations measured at 440 m μ on the proline effluent fractions from the column.

Calculation of amino acid composition

After subtraction of the appropriate "blank" values, the total optical density value for each peak was multiplied by a conversion factor which incorporated the following corrections:

(i) *Different intensity of ninhydrin reaction for each amino acid.* Calibration curves for the ninhydrin reaction with each amino acid were constructed from duplicate assays made at five concentration levels from 1 to 5 μg (approximately) of $\alpha\text{-NH}_2\text{N}$ per ml. The amount of amino acid per millilitre giving an optical density value of 1.0 was calculated for each amino acid and incorporated in the appropriate conversion factor.

(ii) *Percentage recovery of each amino acid from column.* Column chromatography of synthetic amino acid mixtures confirmed the order of elution reported by other workers. Calculation of the percentage recoveries of each amino acid from the column were made and, where necessary, an appropriate multiplier incorporated in the conversion factor.

(iii) *Corrections for serine and threonine.* The values obtained for serine and threonine in the 24 hr hydrolysates were corrected for decomposition by 5 and 3 per cent respectively, as described by EASTOE (1955).

(iv) *Moisture contents.* The moisture contents of each dentine preparation were determined from the losses in weight following drying of the powders overnight in an oven at 110°C ; the appropriate corrections were then included in the hydrolysate calculations.

RESULTS

The plot of a typical elution curve of the amino acid components of a sound dentine hydrolysate, separated on Dowex 50×12 ion exchange columns, is reproduced in Figs. 2 and 3. The results of the analyses of hydrolysates for three sound dentine preparations, three carious dentine preparations and the CD/R fraction from carious dentine, are summarized in Table 1. These results are expressed as grams amino acid residue per 100 g dry dentine preparation, and the total amino acid residue content therefore gives the percentage weight of the dentine preparation accounted for by the analyses. In the sound dentine and carious dentine hydrolysates more or less total content was accounted for (96 and 102 per cent respectively). However, only 86 per cent of the CD/R hydrolysate was accounted for in terms of amino acid content. It has previously been found that the CD/R fraction had a high carbohydrate content (ARMSTRONG, 1960), and analysis of the CD/R fraction used in the amino acid analysis gave a carbohydrate "glucose" value of 13.6 per cent (cysteine method, ARMSTRONG, 1960). With this value added to the 86 per cent amino acid content, 99.6 per cent of the CD/R fraction is accounted for. In column (b) of the CD/R results in Table 1, the amino acid values have been recalculated as percentage values of total protein, assuming a protein content of 86 per cent.

Each preparation showed the characteristic pattern of collagen amino acid composition with its high glycine, proline and hydroxyproline content. Hydroxylysine, though only present in small amounts in collagen molecules, is quite specific and does not appear in any other proteins: it was present in approximately the same concentration in each dentine preparation examined. Although the results showed that carious dentine and the CD/R fraction are essentially collagenous, they also showed that there are differences between the amino acid content of the sound

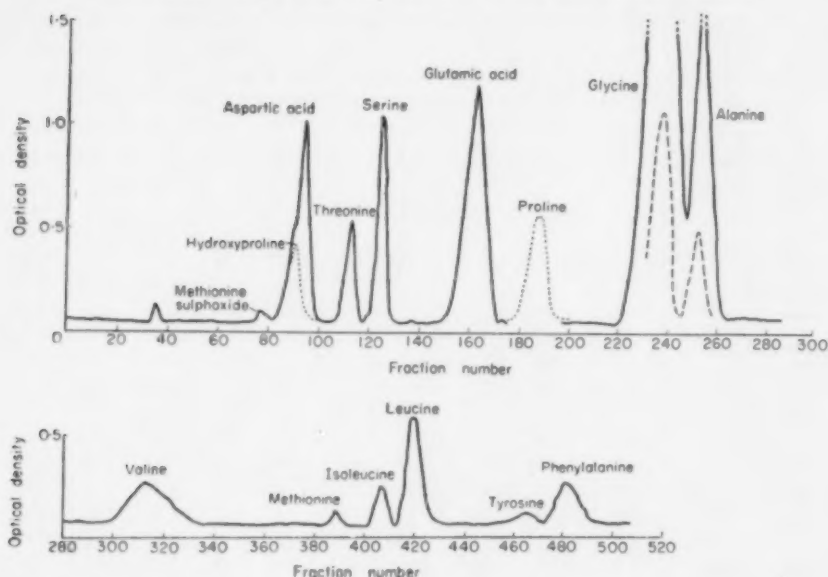


FIG. 2. Elution curve of a sound dentine hydrolysate. Acidic and neutral amino acids separated on 150×0.9 cm column of Dowex 50×12 . —, Optical density/cm at $570 \text{ m}\mu$;, optical density/cm at $440 \text{ m}\mu$; -----, the tops of the glycine and alanine peaks at one quarter vertical scale. Initial buffer (pH 3.41) changed to pH 4.25 at fraction No. 290 and temperature increased from 37 to 60°C .

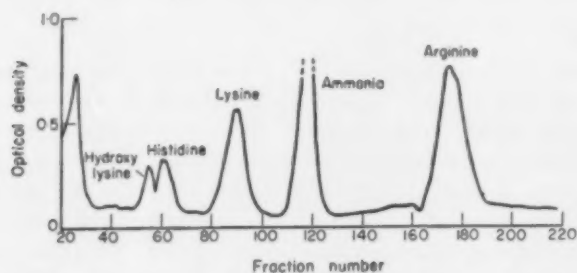


FIG. 3. Elution curve of a sound dentine hydrolysate. Basic amino acids separated on 15×0.9 cm column of Dowex 50×12 . Optical densities/cm measured at $570 \text{ m}\mu$. Initial buffer (pH 5) changed at fraction No. 30 to pH 6.8, and then to pH 6.5 at fraction No. 100, after emergence of lysine peak. The column was run at 25°C throughout.

dentine, carious dentine and CD/R hydrolysates. Comparison of the carious dentine hydrolysates with the sound dentine hydrolysates showed that the carious dentine preparations had a higher content of the following amino acid components: aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. There were possibly slight reductions in the

TABLE 1. AMINO ACID COMPOSITION OF HYDROLYSATES OF DENTINE SAMPLES

Results given as grams amino acid residue per 100 g dry dentine preparation. In column (b) of the CD/R results, the concentrations of amino acids have been recalculated as percentage values of total protein, assuming a protein content of 86 per cent.

Amino acid	Sound dentine				Carious dentine				CD/R	
	1	2	3	Mean	1	2	3	Mean	a	b
Aspartic	7.38	6.27	6.91	6.85	8.88	7.88	9.16	8.64	7.24	8.42
Threonine	1.77	2.31	2.16	2.08	2.72	3.10	3.19	3.00	2.29	2.66
Serine	2.88	2.90	3.22	3.0	4.12	3.88	4.19	4.06	3.23	3.76
Glutamic	10.34	10.44	10.09	10.29	11.75	10.87	11.78	11.47	9.67	11.27
Glycine	17.72	17.64	17.27	17.54	20.95	18.71	18.91	19.52	15.17	17.64
Alanine	8.18	8.29	8.77	8.41	10.64	10.47	11.32	10.81	8.93	10.39
Valine	2.22	2.59	2.68	2.5	3.57	3.80	3.34	3.57	3.26	3.79
Methionine	0.75	0.76	0.62	0.71	1.09	1.06	1.20	1.11	1.23	1.43
Isoleucine	1.37	1.37	1.23	1.33	1.96	1.94	1.86	1.92	1.63	1.90
Leucine	3.16	3.09	3.25	3.17	4.23	4.16	4.75	4.38	3.27	3.80
Tyrosine	0.41	0.49	0.71	0.54	1.71	1.06	1.86	1.47	1.25	1.45
Phenylalanine	1.6	1.83	2.03	1.84	3.35	3.73	3.65	3.57	2.24	2.60
Hydroxylysine	1.05	0.91	1.00	0.99	0.82	0.80	0.98	0.87	0.86	1.0
Histidine	1.01	1.05	1.15	1.07	0.89	0.88	0.86	0.87	1.03	1.2
Lysine	3.28	3.25	3.40	3.31	2.62	3.54	3.67	3.27	3.12	3.63
Arginine	7.8	8.10	7.8	7.9	5.62	6.08	5.59	5.76	3.41	3.97
Proline	13.03	13.2	13.27	13.16	9.86	8.90	9.0	9.25	9.83	11.43
Hydroxyproline	12.03	11.17	12.17	11.79	10.19	9.31	7.55	9.01	8.32	9.67
Total	96.5	95.7	97.7	96.5	105	100.1	102.6	102.5	85.98	100.01

hydroxylysine and histidine content of the carious dentine preparations. Lysine was low in one carious dentine hydrolysate (No. 1) but higher than sound dentine in the other two carious dentine hydrolysates. All three carious dentine preparations showed significantly reduced concentrations of arginine, proline and hydroxyproline. Compared with the carious dentine hydrolysates the CD/R hydrolysate showed reduced concentrations of most amino acids, except proline, which was slightly higher in CD/R. The methionine, hydroxylysine and histidine content was similar in each preparation. Recalculation of the CD/R values to allow for the 13.6 per cent carbohydrate content brought the amino acid content closer to that of carious dentine. However, the arginine content was considerably lower (3.97 per cent) compared with the mean values for sound dentine (7.9 per cent) and carious dentine (5.76 per cent).

DISCUSSION

Before attempting an interpretation of the results of the amino acid analyses reported above, consideration must be given to those modifications which could occur to the organic matrix of the dentine during caries, and could account for the findings.

Various alterations to the collagenous dentine matrix can be envisaged:

I. *No modification occurs to affect the amino acid content in any way.* The amino acid units are the same in carious dentine as in sound dentine, and on hydrolysis yield the same quantities of each amino acid component.

II. *The side-chains of certain amino acid units may be destroyed in situ by some mechanism.* (e.g. the terminal ϵ -NH₂ groups of lysine and hydroxylysine residues removed, or the guanidyl group on arginine side chains removed). In hydrolysates of such modified dentines the unaltered amino acids will be present in the same concentration as in sound dentine, and the amino acids which have been affected will appear in proportionately reduced amounts.

III. *Certain reactive groups of amino acid side-chains may be masked by some reaction during caries.* Upon acid hydrolysis two possibilities may arise:

- (a) The masking radicle may be hydrolysed off and the amino acids will appear in the hydrolysate in the same concentration as in sound dentine. The hydrolysate will be indistinguishable from (I) above, unless the masking substance is a major component of the carious dentine, in which case an overall reduction in all amino acid concentrations may be observed (see V below).
- (b) The hydrolytic procedure itself may cause the masked amino acid units to undergo destruction during, and as a result of, the hydrolysis. The amino acids concerned will appear in lower concentrations in the hydrolysate, which will be indistinguishable from (II) above.

IV. *Certain fragments of the matrix collagen are split off during caries by hydrolytic mechanisms (enzymic or otherwise).* (a) Collagen-like units may be solubilized (e.g. as produced in the gelation of collagen) in which eventuality no difference in amino acid content will be detected in the residual carious dentine matrix.

(b) Alternatively, as the result of enzymic or other processes, peptide units may be split off which have relatively high concentrations of particular amino acid units. The matrix residue will then have lower concentrations of these particular amino acids. As a result hydrolysates will show a general increase in the concentration of the unaffected amino acids and a reduction in content of those amino acids which have been removed in the hypothetical peptide fragments. 100 per cent of the matrix will be accounted for by the amino acid analyses.

V. *Contamination by, or reaction with, non-proteinaceous material (e.g. mono-, di- or polysaccharides).* The result will be reduction in all amino acid concentrations, the percentage reduction being the same for each amino acid. Less than 100 per cent of the total matrix will be accounted for by amino acid analysis alone. This assumes that no destruction of amino acids occurs during the hydrolysis as a result of reaction with non-proteinaceous material. However, the presence of substances, such as carbohydrates, during acid protein hydrolysis may lead to amino acid destruction. If this occurred there would be an overall reduction in amino acids as before, together with a further reduction in those amino acids destroyed during hydrolysis.

VI. *Contamination by, or reaction with, proteinaceous material (e.g. dietary proteins, salivary proteins).* There will be 100 per cent recovery on the total amino

acid basis. However, the carious dentine matrix may show various deviations from the amino acid content of sound dentine, depending upon the nature of the contaminating protein. If this has a high concentration of certain amino acids, which are present in collagen in only moderate amounts, the resultant protein-contaminated carious dentine matrix will give hydrolysates with proportionately higher contents of these amino acids. Conversely, amino acids present in large amounts in collagens, or specific to collagen, will be reduced. Thus significant contamination of collagen by other proteins would also be expected to lead to hydrolysates with reduced concentrations of glycine, hydroxyproline, and hydroxylysine, and probably of proline also.

The six categories discussed above cover the major matrix modifications which are likely to occur. It is probable that more than one effect may occur at the same time.

Examination of results

Examination of the results of the amino acid analyses for the sound dentine, carious dentine and CD/R preparations shows no clear-cut picture as to the nature of the modifications which have occurred in the carious dentine.

The fact that approximately 100 per cent of the carious dentine matrix is accounted for by the amino acid analysis indicates the absence of large amounts of non-proteinaceous material (modification V). However, it is known (ARMSTRONG, 1960) that carious dentine contains approximately 4 per cent carbohydrate, and this value when added to the carious dentine amino acid total gives a recovery mean of 106 per cent.

In the CD/R fraction only 86 per cent of the hydrolysate is accounted for by the amino acid content; a further 13.6 per cent is due to the presence of carbohydrate material.

Examination of the individual amino acid values shows a considerable reduction in carious dentine in the content of the arginine (27 per cent reduction), proline (30 per cent reduction) and hydroxyproline (22 per cent reduction). In the CD/R fraction the arginine concentration is further reduced to a value approximately 43 per cent of that present in the sound dentine matrix. In one hydrolysate (I) the lysine content was low in carious dentine and it was originally thought the modification of the lysine groups had occurred during caries. In hydrolysates 2 and 3, and in other 15 cm short column analyses (unpublished) carious dentine samples have given lysine levels either equivalent to, or slightly greater than those in sound dentine. The shape of such lysine curves often suggested the presence of some other component and it was considered that ornithine, which overlaps with lysine on the short column separations, might be interfering. It was possible that attack on arginine, involving its deguanidation, had led to ornithine formation. However, the use of different buffer systems on the column, which separate ornithine from lysine (HAMILTON and ANDERSON, 1954), failed to indicate the presence of ornithine in carious dentine hydrolysates. This does not exclude the possibility that unidentified components in carious dentine hydrolysates may be artificially raising the lysine levels.

Any extensive proteinaceous contamination at a level sufficient to induce such major reductions in arginine, proline and hydroxyproline levels would be expected also to reduce the high glycine content of the sound dentine, whereas in fact the glycine content of carious dentine is higher by 11 per cent. Similarly the fact that hydroxylysine is only reduced by 11 per cent in carious dentine would seem to indicate that even if this is wholly the effect of proteinaceous contamination, the latter could not account for the greater losses of the arginine, proline and hydroxyproline. Part or all of the hydroxylysine reduction may, of course, be due to some actual destructive modification to the hydroxylysine residues.

It is therefore considered that the results obtained indicate that modifications of Types II, IIIb and/or IVb have probably occurred in carious dentine. However, the fact that all of the carious dentine matrix is accounted for by the analyses indicates that if modifications II and/or IIIb have occurred, this is probably only to a certain limited extent.

In carious dentine there are also large increases in the concentrations of such amino acids as phenylalanine (approximately 100 per cent increase) and tyrosine, which is present at nearly three times the concentration found in sound dentine. These increases are difficult to account for. Most proteins contain less than 10 per cent of each of these amino acids, and to account for the doubling or trebling of their level in carious dentine in terms of contamination by proteinaceous material (modification VI) would involve such an extensive contamination that it would be incompatible with the general collagen-like amino acid pattern found in carious dentine hydrolysates. Similarly, consideration of the extent of proteolytic degradation of the matrix (modification IVb) necessary to cause such changes would require half to two-thirds of the matrix to be solubilized in the form of phenylalanine/tyrosine-free peptide fragments. It is possible that reaction or contamination with phenylalanine/tyrosine-rich peptides (perhaps of bacterial origin) may account for such results. Alternatively, ninhydrin-positive material, either introduced during the carious process or formed by degradation of other amino acid units (arginine, proline, hydroxyproline) may separate on the columns in the phenylalanine and tyrosine positions, so artificially raising the concentrations recorded for these components.

In connexion with relatively smaller differences in the concentration levels of other amino acids, it is difficult to establish if these concentration shifts are due to protein contamination (modification VI) or to the removal of soluble peptide fragments from the matrix (modification IVb). However, as discussed above, the fact that the glycine content of carious dentine is high, coupled with the comparatively high hydroxylysine content, makes it unlikely that gross contamination by non-collagenous protein has occurred during caries. On theoretical grounds it seems very likely that both types of modification might occur in the carious process in dentine.

A direct comparison of the CD/R fraction analysis with those of sound and carious dentine is complicated by the possibility that the collagenase action may have also introduced Type IVb modifications of the matrix. Nevertheless, it is apparent that the corrected amino acid content of CD/R is similar to that of carious dentine, and that a higher carbohydrate content and a lower arginine content is associated

with (though not necessarily causally related to) the total resistance to collagenase disintegration.

In summary, modifications occur to the dentine matrix during caries which result in altered amino acid concentrations in carious dentine hydrolysates. The major features are the reduction in arginine, hydroxyproline and proline, and it is likely that these changes reflect intrinsic modifications to these amino acid residues in the matrix during caries. There is evidence that contamination by non-collagenous protein and proteolytic degradation has occurred to the matrix to a certain extent during the carious process. Further, the partial resistance to collagenase attack of carious dentine and the total resistance of the CD/R fraction is paralleled by an increased carbohydrate and lowered arginine content.

It is to be noted that *in vitro* modifications to dentine collagen, involving destruction or blocking of the guanidyl groups of arginine side-chains, or of the hydroxylysine groups of amino acid residues, will convert collagen to collagenase resistant forms (ARMSTRONG, 1958b). Since the carious dentine amino acid analyses indicate that modifications involving these amino acid residues have probably occurred in carious dentine, this could therefore provide an explanation of the collagenase-resistant property of carious dentine.

The possibility that the presence of carbohydrate in carious dentine and the CD/R fraction may also be related to the collagenase resistant properties of these samples has been considered, and the results of certain *in vitro* investigations into this possibility are reported in a further communication (ARMSTRONG, 1961).

Acknowledgement—I am greatly indebted to Miss BRITA ERHOLTZ for her excellent technical assistance throughout this investigation.

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DENTAL CARIES AND MOTTLED ENAMEL AMONG FORMOSAN CHILDREN

M. Y. PU and B. LILIENTHAL*

Kaohsiung City Health Centre, Taiwan, and Department of Oral Medicine and Surgery, University of Melbourne, Australia

Abstract—A survey of dental caries and mottled enamel was carried out among Formosan children. The prevalence of dental caries was approximately half that of western civilized countries. In areas where the water contained fluoride naturally, the prevalence of dental caries was inversely related to the fluoride content of the water supply. Mottled enamel was prevalent and to a greater extent than could be accounted for by the fluoride content of the water consumed. The relation between dental caries and mottled enamel was examined in connexion with the most suitable fluoride level to be employed in an artificially fluoridated water supply. The contribution of fish to the fluoride intake is discussed and the value of reducing the prevalence of dental caries in less developed countries is emphasized.

INTRODUCTION

THE ISLAND of Formosa lies approximately 100 miles off the south coast of China and is separated from it by the Formosan Straits. Shaped somewhat like a tobacco leaf it is about 230 miles from north to south and some 90 miles across at its greatest width. Only one third of the country is suitable for agriculture and the population of ten million live mainly on the eastern and western coastal plains which are separated by a mountainous region rising to 13,000 ft and running from north to south. The plains are watered by twelve streams which flow rapidly over the steep gradients in the rugged upper terrain but sluggishly through the plains, where they are to a greater or lesser extent diverted to irrigation canals for agricultural use.

The Tropic of Cancer passes through Formosa and its climate is of a tropical nature with mean annual temperature approximately 75°F. and 80 per cent humidity. The island lies in the Western Pacific typhoon zone and rainfall averages about 100 in. per annum.

Nearly 30 per cent of the population is served by communal water supplies either large or small. In the rural areas there are nearly 13,000 public wells and some 130,000 private wells from which another 45 per cent of the population derive their water supplies. The remaining 25 per cent use water from rivers, ponds, canals or rain water catchments (YUNG, 1957).

The chief crops are rice, sugar cane and sweet potatoes. An abundance of vegetables is grown and such fruits as bananas, oranges, pineapples, water melons and

* Present address: School of Bacteriology, University of Melbourne, Victoria, Australia.

papayas. Rice is the main dietary staple with an annual consumption of about 180–200 kg per person. Rice, by law, is of 93 per cent extraction and as it enters the debranning machine, 1–2 per cent calcium carbonate is added to the brown rice, to give it a white appearance.

An estimate of the nutrient composition of the dietary is necessarily less accurate than for other countries because no food consumption tables are available giving the nutrient values of Formosan foods. Furthermore, food consumption is at best a guess for rural communities which comprise 75–80 per cent of the population. However, JOLIFFE and TUNG (1956) claim that the diet throughout the island is remarkably uniform. Exceptions quoted by them included 100,000 aborigines who use rice, chestnuts, taro, millet and sweet potato as their staple diet; a small group which, because of crop conditions in an arid portion of the island, uses the sweet potato instead of rice; a group of Northern Chinese who mostly eat wheat as their main foodstuff; and a relatively small upper income group. These groups together comprise not more than 10 per cent of the civilian population.

In a survey of the nutritional status of about 1000 sixth grade school children, JOLIFFE and TUNG (1956) concluded that several types of malnutrition were prevalent (Table 1). Some improvement in general nutrition has taken place since their survey.

TABLE 1. NUTRITIONAL DEFICIENCIES AMONG 1000 CHILDREN
IN FORMOSA (6TH GRADE)
(JOLIFFE and TUNG, 1956)

Substance or disease	Sufferers (%)
Riboflavin	70
Low excretion of urinary thiamine	55
Anaemia (iron deficiency)	13
Vitamin A	10
Niacin	10
Hypoproteinaemia	3.2
Beri-beri	0

Dietary habits of importance in relation to dental caries include the regular consumption of readily fermentable carbohydrates of which "sweets" and "candy", biscuits, jams, etc. are the commonest examples. Sweets and candy-type articles were for sale in the shops but the economic status of the family unit was such that these items of diet appeared to be of only minor importance. Sugar cane sticks were relatively cheap in the area where most of the survey was carried out but no information was obtainable which would indicate how important this might be in relation to dental caries.

Between Formosa and China lies the Pescadores group of islands. These are a low, arid, windswept and almost treeless cluster of small islands of which only some are inhabited. In two villages here and in parts of southern Formosa, there are well-waters known to contain fluoride up to as much as 1.6 p.p.m. One of us (M.Y.P.)

first discovered mottled enamel in teeth of children in Southern Formosa and recognized also similar conditions amongst children who had migrated from the Pescadores to the city of Kaohsiung in Formosa. The distribution of fluoride-containing well waters has not yet been extensively explored but the places now known to have up to 1.6 p.p.m. fluoride are shown in Fig. 1.

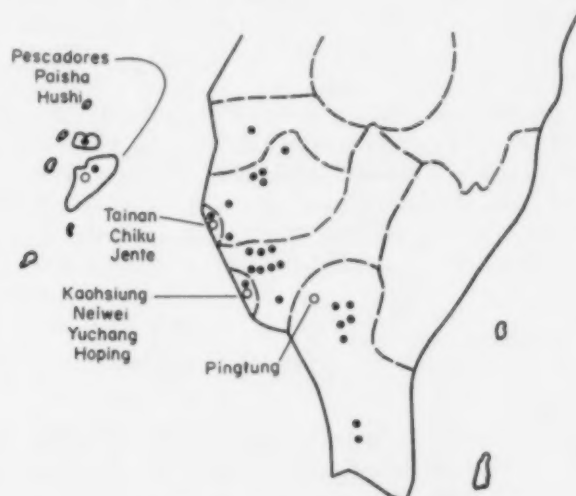


FIG. 1. Distribution of areas of mottled enamel in Southern Formosa.

PURPOSE OF DENTAL SURVEY

The dental survey reported here was carried out during October–November 1958 and is the first extensive survey of the prevalence of dental caries and mottled enamel among Formosan children. It was undertaken to obtain information relevant to the optimal fluoride values to be maintained in an artificially fluoridated water supply in Formosa where the mean annual temperature is high, the diet markedly different from that of the western civilized countries and where the intake of fluoride in food, as distinct from in drinking water, might be a significant factor in determining the optimal value.

METHODS

Plan of survey

Throughout the survey attempts were made to examine as large a number as possible in each selected age group. The dental examinations were made in good natural light with mouth-mirror and sharp standard right angle or sickle explorers. Occlusal caries was determined by the tendency of the probe to "stick in the fissure". Details of age, sex, type of water supply, DMF and def teeth and mottled enamel were recorded for each child. Children aged 6, 8, 10 and 11 years at last birthday were examined in several districts.

Mottled enamel was recorded essentially as described by DEAN (1942). Grades nil, F1, F2, F3 and F4 were employed, the criteria for which were as follows:

Nil—normal; no evidence of abnormality.

F1—fine white lines or small white flecks on the enamel surfaces.

F2—single or multiple white lines or bands 0.5 mm or more in width.

F3—generalized white appearance of the enamel or very large white patches.

F4—as for F3 with the additional features of pitting (hypoplasia).

The community fluorosis index

DEAN (1942) described an index which suitably expresses the prevalence and severity of mottled enamel in a community. He assigned numerical values to each grade of mottled enamel as follows: questionable, 0.5; very mild, 1.0; mild, 2.0; moderate, 3.0; and moderately severe, 4.0. The corresponding values in this report are: F1=0.5, F2=1.0, F3=2.0, F4=3.0. The community index is calculated from the frequency of each grade, its statistical weight or value and the number in the group, thus:

$$\text{community index} = \frac{\text{frequency} \times \text{statistical weight}}{\text{number examined}}$$

Examiner calibration

Before starting the survey the authors separately examined the same group of 82 children (Table 2). A difference of approximately 5 per cent was found between the examiners for DMF teeth. The mottled enamel scoring was also of the same order. To minimize the effect of such differences as existed between the examiners, throughout the survey each of us examined half of each group of boys and of each group of girls. At the end of the survey a final check was made by each of us examining the same group of 84 children (Table 2). Therefore, it can be concluded that two

TABLE 2. CALIBRATION OF EXAMINERS

Initial calibration

Examiner	No. in group	No. DMF teeth	Mottled enamel (Grade)				
			Nil	F1	F2	F3	F4
A	82	242	52	16	11	3	—
B	82	226	57	16	6	3	—

Final calibration

Examiner	No. in group	DMF teeth (First molars)			Mottled enamel (Grade)			
		Max.	Mand.	Total	Nil	F1	F2	F3
A	84	48	111	159	60	19	5	—
B	84	54	108	162	60	21	3	—

examiners had similar criteria for judging dental caries and mottled enamel and that the error derived from differences between the examiners remained constant throughout the survey.

Water analyses

The values quoted in the tables for fluoride ion in the water are those obtained by analysis prior to this survey. Analyses of well-waters and town supplies during the survey agreed substantially with these values.

However, at Neiwei the children were either brought up on the town water supply or drank water from wells. Analysis of water from 115 wells in this area showed a wide range of fluoride ion values, approximately 42 per cent of which were 0.5 p.p.m. or more, ranging up to five waters with 1.1 p.p.m. 50 per cent of fluoride values were between 0.2 and 0.4 p.p.m. inclusive.

At the villages of Yuchang and Hoping no water samples contained more than 0.4 p.p.m. fluoride ion.

The values of 0.5 p.p.m. for Hushi and Paisha in the Pescadores were verified.

RESULTS

1. Prevalence of dental caries

The carious lesions were generally confined to the premolar and molar teeth, especially the latter, and were characteristically of the pit and fissure type. When the incisor teeth were involved the sites attacked were the lingual pits and not the smooth surfaces. The figures given for DMF teeth (Tables 3-6) consist almost

TABLE 3. DENTAL CARIES PREVALENCE DATA
(11 YEAR-OLDS)

School	No. in group	DMF teeth	DMF per child	Fluoride (p.p.m.)
Formosa				
Neiwei	60	230	3.83	Nil
Yuchang } Hoping }	125	272	2.18	0.2-0.4
Neiwei	76	149	1.96	0.6-0.9
Chiku	180	245	1.36	0.85
Jente	137	179	1.30	1.6
Pescadores				
Hushi	182	100	0.55	0.5
Paisha	91	54	0.59	0.5

entirely of decayed untreated lesions, there being very few teeth either missing or with restorations among those examined. An average DMF teeth per child of 3.8 was found for 11 year-olds who drank fluoride-free water. The values at 10 and 8 years were 1.8 and 1.7 respectively. These values compare very favourably with

corresponding age groups in western-civilized countries, e.g. Australia, where at 11, 10 and 8 years 7.0, 5.3 and 3.2 DMF teeth per child were found by BARNARD (1956).

TABLE 4. DENTAL CARIES PREVALENCE DATA
(10 YEAR-OLDS)

School	No. in group	DMF teeth	DMF per child	Fluoride (p.p.m.)
Formosa				
Neiwei	29	52	1.79	Nil
Yuchang	109	177	1.62	0.2-0.4
Hoping				
Neiwei	71	112	1.58	0.6-0.9
Chiku	64	76	1.19	0.85
Jente	105	108	1.03	1.6
Pescadores				
Hushi	47	23	0.49	0.5
Paisha	50	33	0.66	0.5

TABLE 5. DENTAL CARIES PREVALENCE DATA
(8 YEAR-OLDS)

School	No. in group	DMF teeth	DMF per child	Fluoride (p.p.m.)
Neiwei	46	77	1.67	Nil
Yuchang	89	143	1.61	0.2-0.4
Hoping	76	114	1.50	0.2-0.4
Neiwei	73	67	0.92	0.6-0.9

TABLE 6. DENTAL CARIES PREVALENCE DATA
(6 YEAR-OLDS)

School	No. in group	def teeth	def per child	Fluoride (p.p.m.)
Neiwei	76	565	7.43	Nil
Yuchang	114	1059	9.29	0.2-0.4
Hoping	65	609	9.21	0.2-0.4
Neiwei	51	233	4.57	0.6-0.9

The number of carious mandibular molars was significantly greater than their maxillary counterparts (Tables 7 and 8).

TABLE 7. PREVALENCE OF DENTAL CARIES IN FIRST PERMANENT MOLARS (11 YEAR-OLDS)

School	No. in group	DMF MI			DMF MI per child			Fluoride (p.p.m.)
		Max.	Mand.	Total	Max.	Mand.	Total	
Formosa								
Neiwei	60	59	89	148	0.98	1.48	2.47	Nil
Yuchang	78	41	89	130	0.53	1.14	1.66	0.2-0.4
Hoping	47	30	50	80	0.64	1.06	1.70	0.2-0.4
Neiwei	76	35	74	109	0.46	0.97	1.43	0.6-0.9
Chiku	180	50	149	199	0.28	0.83	1.10	0.85
Jente	137	35	104	139	0.25	0.76	1.01	1.6
Pescadores								
Hushi	182	12	67	79	0.07	0.37	0.43	0.5
Paisha	89	8	39	47	0.09	0.43	0.52	0.5

In the deciduous dentition the prevalence of dental caries was higher in the non-fluoride or low fluoride areas. The average def/child was between 7 and 9 approximately in these areas of Formosa (Table 6).

TABLE 8. PREVALENCE OF DENTAL CARIES IN FIRST PERMANENT MOLARS (10 YEAR-OLDS)

School	No. in group	DMF first molars			DMF first molars per child			Fluoride (p.p.m.)
		Max.	Mand.	Total	Max.	Mand.	Total	
Formosa								
Neiwei	23	10	31	41	0.43	1.35	1.78	Nil
Yuchang	69	24	68	92	0.35	0.98	1.33	0.2-0.4
Hoping	41	23	47	70	0.56	1.15	1.71	0.2-0.4
Neiwei	71	20	77	97	0.28	1.08	1.36	0.6-0.9
Chiku	64	16	45	61	0.25	0.70	0.95	0.85
Jente	105	16	68	84	0.15	0.65	0.80	1.6
Pescadores								
Hushi	47	3	18	21	0.07	0.38	0.45	0.5
Paisha	50	4	25	29	0.08	0.50	0.58	0.5

The frequent observation of a slightly greater prevalence of dental caries in females than in males was also found among these Formosan children although the data on this aspect have not been included.

2. The incidence of dental caries

The annual rate of increase in dental caries in the permanent dentition was found to be approximately 0.7 DMF teeth per child in fluoride-free areas. This value for the incidence of dental caries is only half that found in Australia where the annual

increase in DMF teeth per child is nearer 1.5. The incidence was much lower in areas where fluoride-containing water was consumed (Table 9).

TABLE 9. INCIDENCE OF DENTAL CARIES

School	Fluoride (p.p.m.)	Age last birthday (years)			DMF per year 8-11 years)
		8	10	11	
Neiwei	Nil	1.67	1.79	3.83	$\frac{3.83-1.67}{3} = 0.72$
Yuchang Hoping }	0.2-0.4	1.56	1.62	2.18	$\frac{2.18-1.56}{3} = 0.21$
Neiwei	0.6-0.9	0.92	1.58	1.96	$\frac{1.96-0.92}{3} = 0.31$

3. Prevalence of dental caries in natural fluoride areas

The results of examination of 11, 10, 8 and 6 year-old children are shown in Tables 3-8. In general the prevalence of dental caries by age decreased as the fluoride content of the drinking water increased.

4. The prevalence and severity of mottled enamel

The distribution of mottling in the tooth enamel was typical of this condition. Brown staining was not a regular or marked feature and the striations were essentially white. Idiopathic white spots on the enamel appeared to be relatively uncommon. The fluorosis index for the children at Neiwei school who drank only fluoride-free water suggests a significant distribution of very mild mottled enamel possibly caused by the fluoride intake in food.

TABLE 10. PREVALENCE OF MOTTLED ENAMEL

School	No. in group	Number of children showing fluorosis (Grade)					Fluoride in water (p.p.m.)	Community fluorosis index
		Nil	F1	F2	F3	F4		
Formosa								
Neiwei	89	75	10	4	—	—	Nil	0.10
Yuchang	147	104	31	11	1	—	0.2-0.4	0.26
Hoping	88	49	31	8	—	—	0.2-0.4	0.27
Neiwei	147	60	59	21	7	—	0.6-0.9	0.44
Chiku	244	42	88	92	22	—	0.85	0.74
Jente	242	38	51	89	58	6	1.6	1.02
Pescadores								
Hushi	229	33	76	77	43	—	0.5	0.88
Paisha	139	52	49	30	7	1	0.5	0.51

The results of observations on mottled enamel are shown in Table 10. The increase in severity of mottled enamel with increased fluoride content of the water supplies is apparent and with 0.85 p.p.m. the number of children (22) showing Grade F3 mottled enamel is sufficient to indicate that this level is too high for use in artificially fluoridated water projects in this region.

Calculation of the community index gave the values shown in Table 10. A concentration of fluoride ion between 0.4 and 0.8 p.p.m. offers, for Formosa, a satisfactory reduction in the prevalence of dental caries, at the same time producing a minimal degree of mild mottling of the enamel.

5. Diet and mottled enamel

At Hushi and Paisha in the Pescadores, where only 0.5 p.p.m. fluoride was present in the well water, quite different results were obtained. The proportion of children with a moderate degree of mottled enamel was high and the community index was 0.88 and 0.51 respectively. The "fluorosis index" in these villages is higher than can be explained by the 0.5 p.p.m. fluoride found in the drinking water and enquiries were made concerning the diet. The villagers were dependent on fish and sweet

TABLE 11. POSSIBLE FLUORIDE CONTRIBUTION FROM DIET AND WATER
IN THE PESCADORES

Fluoride from 1.0 lb fish at		Fluoride from water (0.5 p.p.m.)	
10 p.p.m.	15 p.p.m.	1 l.	1.5 l.
0.91 mg	1.37 mg	0.5 mg	0.75 mg

potatoes for their basic diet and the children daily had as much as 1 lb or more of fish of smaller varieties whose skeleton is also consumed. Estimates of the fluoride content of the fish component of the diet of these children suggest that approximately 1-1.5 mg might be provided by the diet from 1 lb of fish as compared with 0.5-0.75 mg from water (Table 11).

The diet, therefore, provides more fluoride than the water consumed.

6. Observations on dietary patterns

An excessive consumption of fermentable carbohydrate is reflected by the oral flora showing a high lactobacillus count and by Snyder tests with grades 2+, 3+ and 4+. Using the hydroxyapatite agar plate, LILIENTHAL and REID (1959) showed that adaptation to sucrose fermentation occurs when sucrose is regularly consumed. Both techniques were employed to assess the difference, if any, in the dietary pattern of children who were using (and always had used) a fluoride-free or fluoride-containing water supply.

The results show that the percentage of negative and grade+ Snyder tests increased with the increase in fluoride content of the water supply (Table 12). Contrary to this was the similar distribution of sucrose adaptation found among the

TABLE 12. COMPARISON OF SNYDER TESTS OF SALIVA FROM CHILDREN OF FLUORIDE-FREE AND FLUORIDE AREAS

Area	Fluoride in water (p.p.m.)	No. in group	Snyder tests (grade)	
			- or +	2+, 3+, 4+
Neiwei	Nil	79	34 (48%)	41 (52%)
Yuchang	0.2-0.4	118	64 (54%)	54 (46%)
Neiwei	0.6-0.9	116	77 (66%)	39 (34%)

Results significant at $P=0.05$.

TABLE 13. ADAPTATION TO SUCROSE IN SALIVA

Area	Fluoride in water (p.p.m.)	No. in group	Adaptation to sucrose*
Neiwei			
Town supply	Nil	50	22
Wells	0.6-0.9	50	18

Results not significant at $P=0.05$.

* Adaptation to sucrose was recorded when the diameter of clear zone using sucrose-saliva ≥ 1 mm greater than diameter of the clear zone using glucose-saliva.

fluoride-free and fluoride groups at Neiwei (Table 13). There was no major difference between the groups, supporting the view that the diet of each group was similar as regards consumption of sucrose containing foods.

DISCUSSION

The results of the survey show a dental caries prevalence approximately 50 per cent lower than is general in western-civilized countries and a distribution of the lesions essentially confined to the pits and fissures especially of the molar teeth. Lack of oral hygiene measures, such as the use of a toothbrush, was general among these children and probably contributed to the prevalence of the lesions and their particular location. This is supported by the marked difference between dental caries of the maxillary and mandibular molars which was associated with an obviously greater accumulation of food debris around the latter.

In the natural fluoride areas there was a general decrease in the prevalence of dental caries with increased fluoride in the water. In some instances the number in the fluoride-free group is small, thus allowing only general trends to be shown. Percentage reduction in dental caries, therefore, has not been emphasized. However, in spite of the variable fluoride content of the well waters in Neiwei it may be concluded that approximately 50 per cent reduction in dental caries was attained in the 0.6-0.9 p.p.m. group. At Chiku where 0.85 p.p.m. fluoride was found, the reduction in dental caries was greater, approximately 65 per cent for 11 year-olds.

With the exception of the villages in the Pescadores the diet of the children was basically the same. The accumulation of starch in the mouth is the likely source of

fermentable material associated with the formation of carious lesions. The limited tests at Neiwei supported the view that other sources of fermentable carbohydrate, such as sucrose, were evenly distributed among children drinking fluoride-free or fluoride-containing waters. It may be concluded that dietary difference between these groups were not of major importance and, therefore, the change in Snyder tests, which represents a decrease in lactobacillus counts in the salivas of children in fluoride areas, were the result of a lower prevalence of dental caries and not due to a different dietary pattern in the fluoride areas.

In the villages of Hushi and Paisha in the Pescadores the prevalence of dental caries was very low indeed and this was undoubtedly the result of a combination of fluoride intake during tooth development and a minimum consumption of fermentable carbohydrate other than starch. Here, also, starch (rice) was not so readily available.

The prevalence and intensity of mottled enamel was similar to other countries if suitable allowance for increased fluid intake in a region with high mean annual temperature and humidity is made. An important exception was found in the Pescadores at the villages of Hushi and Paisha where the amount of mottling was more than could be accounted for by the fluoride content of the well-water consumed. The enquiries made into the possible sources of fluoride showed clearly that water was neither the sole nor the major source of fluoride intake and led to a consideration of the contribution which sea-food, especially fish, could make to the fluoride intake.

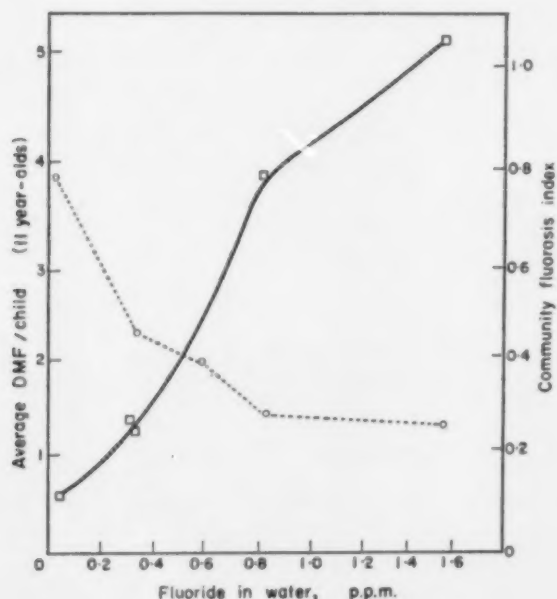


FIG. 2. The relation between dental caries, mottled enamel and fluoride content of water in Formosa.

It is obvious that at least as much fluoride daily could be taken in with food as with water. The supporting evidence, although an approximation, illustrates the probable source of fluoride and gives an explanation for the severity of mottled enamel in these villages.

It would appear that in Formosa 0.6 p.p.m. fluoride would be sufficient to attain a 50-60 per cent reduction in the prevalence of dental caries in this region and a satisfactory fluorosis index (Fig. 2). In the Pescadores, where mottled enamel is the result of a combination of a relatively large fluoride intake with food in addition to water-borne fluoride, de-fluoridation of the water would be necessary to reduce the severity of mottled enamel if an alternative food supply was not obtainable.

This survey indicates the need for a careful assessment of dietary sources of fluoride in countries where the dietary pattern is not similar to that in the U.S.A. and other western-civilized countries. This is especially important in communities where fish and sea-foods are important items of the diet.

The part which fluoridation of communal water supplies could play in reducing the prevalence of dental caries is very important to the development of dental health in underdeveloped countries. In Formosa, where a population of ten million people is served by approximately two hundred qualified dentists, the prevention of dental caries by fluoride-containing water supplies would efficiently and cheaply overcome some of the difficulties in providing the population with dental services.

Acknowledgements—Our thanks are due to the Regional Director of the Western Pacific Region of the World Health Organization and to the Minister for Public Health of the Government of Nationalist China for permission to publish the results of this survey. This paper is based on a report by LILIENTHAL (1958) for the World Health Organization.

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THE MAIN MINERAL CONSTITUENT OF BONE AND TEETH

HUGUETTE HERMAN and M. J. DALLEMAGNE

Institut de Thérapeutique Expérimentale, Université de Liège,
Belgique

Abstract—Pyrophosphate was found in heated cow bone and dentine and in elephant enamel. The influence of heating on these materials was studied and compared to the behaviour of mixtures of hydrated tricalcium phosphate (TCPH) and CaCO_3 in the same conditions.

The amount of pyrophosphate found in these mixtures depended on the degree of homogeneity. If the organic fraction of bone was removed, the mineral part behaved as a homogeneous mixture of synthetic TCPH and CaCO_3 . It is concluded that the mineral fraction of these tissues contains an apatitic phosphate with a Ca/P ratio lower than hydroxyapatite. It is suggested that the organic phase of bone, dentine and enamel plays a part in preserving this compound from the extra calcium belonging to a non-phosphatic mineral fraction.

INTRODUCTION

IT HAS BEEN recently shown that hydrated tricalcium phosphate (TCPH) and the other apatitic calcium phosphates with a Ca/P weight ratio lower than 2.15 contain acid phosphate groups linked by hydrogen bonds ($\text{O}_3\text{PO}-\text{H}-\text{OPO}_3$) and located in the lattice. Two adjacent unit cells liberate one constitutive H_2O molecule when heated to 600°C or less, giving one pyrophosphate group ($\text{P}_2\text{O}_7^{4-}$) which may be detected by chemical methods. The pyrophosphate groups of TCPH disappear over 700°C when β -tricalcium phosphate is formed and a second constitutive H_2O molecule is lost (per two unit cells). This water which is absolutely different from external adsorbed water (quantitatively depending on the Ca/P ratio of the compounds) has been detected on infra-red spectrograms (principally, 2400 cm^{-1}) and quantitatively determined by thermogravimetric methods.

Detailed data concerning the structure of these compounds have been published elsewhere (WINAND, 1960); in fact, an extensive study of apatitic phosphates with various Ca/P ratios and of TCPH and CaCO_3 (or CaO) mixtures has been made by HERMAN, FRANÇOIS and FABRY (1961). These authors have principally shown that the amount of pyrophosphate found after heating is inversely proportional to the Ca/P ratio of these compounds. They have pointed out, confirming the findings of Winand, that conventional hydroxyapatite (Ca/P weight ratio = 2.15) does not contain pyrophosphate after heating. On the other hand, according to the same authors, a biphasic system, for instance TCPH and CaCO_3 , with a Ca/P ratio of 2.15 or more, still gives pyrophosphate after heating provided that the excess calcium pertaining to the non-phosphoric phase cannot react with the phosphoric phase during the

thermal treatment. In this way, the presence of TCPH or of low Ca/P ratio apatitic calcium phosphate in such physical mixtures may be detected by experimental determination of pyrophosphates.

Therefore, this method provides a means of demonstrating the presence of such a calcium phosphate in bone and teeth in spite of the high Ca/P ratio of the mineral of these tissues; preliminary results indicate that at least a part of the non-apatitic excess calcium is in some way kept apart from the main phosphoric constituent of bone salts (FRANÇOIS and HERMAN, 1961). The amount of pyrophosphate found in heated bone mineral depends on the age of the animal and is influenced by the diet (FRANÇOIS, 1961).

These studies have been extended to dentine and enamel; the purpose of this paper is to compare the results obtained with dental tissues, bone and synthetic phosphates, in order to throw some light on the molecular constitution of the mineral phase of these biological materials.

METHODS

The chemical methods used have been described in a previous publication (HERMAN *et al.*, 1961), so we shall only summarize some important features of our techniques.

Preparation of the material studied

(1) TCPH was prepared by adding the stoichiometric amount of $\text{Ca}(\text{OH})_2$ to diluted H_3PO_4 at 20°C.

(2) The compounds with a higher Ca/P ratio were prepared by WALLAEYS' method (1952), by mixing the stoichiometric amounts of $\text{Ca}(\text{OH})_2$ and H_3PO_4 at 100°C. Hydrated hydroxyapatite precipitate was obtained by the same method (Ca/P weight ratio=2.15). In this way, all the Ca ions are introduced into the lattice of the different compounds.

(3) Mixtures of TCPH (dried at 105°C) and CaCO_3 or CaO (particles passing Tyler mesh 42) were made

(a) in a micromill (Glen Creston) for 10 min, which gives coarse mixtures (FABRY, 1961) or

(b) in a Heidelberg's colloidal ball mill (COGHILL and DEVANEY, 1937) which is much more efficient (FABRY, 1961); by increasing the grinding time in the agate vessel of the latter, the crystals are crushed and more and more intimate mixtures are obtained.

(4) Bone from an adult cow was defatted in carbon tetrachloride and ground in the mineralogical Abich's mortar. In some experiments the particles were ground again in the colloidal mill and dried at 105°C, or ashed by the GABRIEL's method (1894) (KOH-glycerol was removed by water or alcohol washings).

(5) Dentine from the teeth of cows was treated in the same manner.

(6) Elephant enamel was carefully isolated from dentine and cementum and purified by flotation in a bromoform-alcohol mixture of density 2.5.

Heating and analysis methods

The samples were heated in an electric oven for various times (1–150 hr); the accuracy of temperature measurements was $\pm 20^\circ\text{C}$.

After this treatment, the sample of mineral (still containing some carbon in the case of bone and dentine) was dissolved in 0.01 N HCl and the solution was allowed to flow on an Amberlite IR 400 column in order to fix ortho- and pyrophosphates (FRANÇOIS, 1960; WINAND, 1960): the former were eluted by 0.05 N HCl and the latter by N HCl. Pyrophosphates were measured after hydrolysis by the phosphorus method previously described (FISKE and SUBBAROW modified by ALLEN, 1940).

It must be pointed out that the Ca/P weight ratio of the compounds is established with a standard error of ± 0.8 per cent and that the theoretical amount of pyrophosphate that should be found is calculated from this ratio. The determined amount of pyrophosphate is the mean value of two or three chromatographic separations giving satisfactorily close results.

RESULTS

Quantitative results are summarized in Table 1 and Fig. 1, including, for comparison, some previously published figures (FRANÇOIS and HERMAN, 1961).

TABLE 1. PER CENT PYROPHOSPHATE AFTER 1 hr HEATING

	Ca/P	% max. theor.	105 °C	200 °C	250 °C	300 °C	325 °C	350 °C	400 °C	450 °C	500 °C	600 °C	700 °C	900 °C
TCPH	1.93	17.13	0.25	2.30		10.31			12.65		15.64	15.56	1.72	
Wallaeys synth. phos.	2.08	5.24	0.02	0.50		1.32			2.11		4.99	5.53	0.61	0.09
	2.16	0		0.30					0.90		0.81	0.38	0.17	0.10
	2.20	0	0.05	0.10		0.33			0.25		0.27	0.10	0.10	0.02
TCPH + CaCO ₃ ground for 20 hr in a colloidal mill	2.00	11.90		1.44	3.30	5.38		9.42	10.88	12.51	11.97	9.86		
	2.15	0		1.07		4.89		6.62	9.44	8.90	8.24	2.95		
	2.25	0		0.81	2.20	5.10		6.70	7.65	3.65	0.99	0.25		
Total cow bone	2.18	0	0.62	0.40	0.96	3.42	4.40	3.42	2.09		0.81			
Total cow dentine	2.13	1.35	0.57	0.58	2.47	3.99	4.36	4.07	3.12		2.32			
Total elephant enamel	2.12	2.38	0.10	0.42		0.78			1.57		2.14	2.21	0.73	

1. Influence of heating on the amount of pyrophosphate

All the samples were heated in air for 1 hr at various temperatures increasing from 105 to 900°C. When biological materials were used, we found a small amount of pyrophosphates after drying at 105°C; presumably it corresponds to the pyrophosphoric phase described by CARTIER (1959) and is derived from organic compounds. The amount of pyrophosphate increased with the temperature of heating and reached a maximal value at a temperature level which depended on the nature

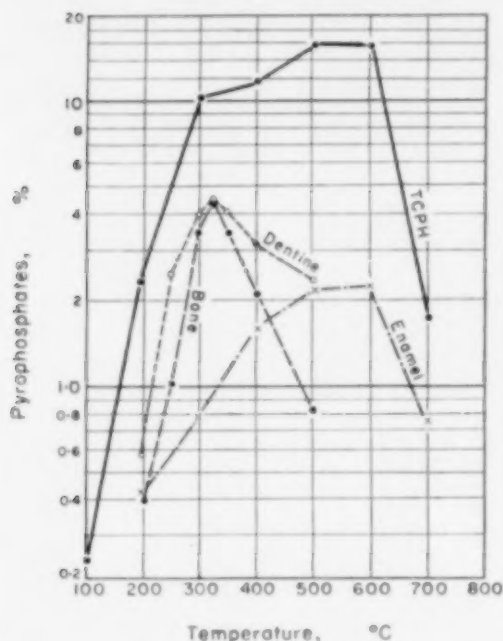


FIG. 1. The effect of heating on the amount of pyrophosphate in hydrated tricalcium phosphate (TCPH), bone, dentine and enamel. A semi-logarithmic scale has been used only to make the graph more obvious.

of the considered material: this level is around 600°C for TCPH and the phosphates with a higher Ca/P ratio, all the Ca ions being present in the lattice (WALLAEYS' synthetic phosphates). When mixtures of TCPH and CaCO_3 (ground for 20 hr in the colloidal mill) were considered, as the CaCO_3 amount increased, so the temperature giving the maximal yield of pyrophosphate decreased to 450–400°C. In the case of bone and dentine, this temperature was still lower and decreased to 325°C. The behaviour of enamel was the same as observed for synthetic TCPH and Wallaeys' apatitic phosphates and the maximal yield of pyrophosphates appeared at 500–600°C.

2. Influence of the Ca/P ratio on the amount of pyrophosphate

The amount of pyrophosphate decreased when the Ca/P ratio of the Wallaeys' compounds increased, corresponding rather well to the values calculated from the Ca/P ratio. It also decreased when TCPH and CaCO_3 or CaO mixtures are concerned, but in this case the amount of pyrophosphate found was much higher than it could theoretically be calculated from the Ca/P ratio of the mixture taken as a whole: only a part of the added calcium of CaCO_3 or CaO may react with the acid phosphate or the pyrophosphate groups during the thermal treatment and enter into the lattice to form hydroxyapatite.

3. Influence of the heating time

If the heating time was longer than 1 hr, the amount of pyrophosphate present in the compounds with a Ca/P ratio higher than 1.94 (Wallaeys' compounds), did not change. It was always lower after heating at 300°C than after treatment at 600°C and the disappearance of pyrophosphate never began under 700°C even if the compound was heated for 110 hr (Table 2).

TABLE 2

	Ca/P	Heating temp. (°C)	Heating time (hr)				
			1	15	85	110	140
TCPH	1.93	300		10.31	10.54	10.49	
Wallaeys synth. phos.	2.08	600		5.53		5.36	
TCPH + CaCO ₃	2.00	450		12.51		11.64	
	2.15	400		9.44		5.97	
	2.25	400		7.65		3.28	
Total cow bone	2.18	300	3.42	3.48	2.62		2.27
Total cow dentine	2.13	325	4.36	4.29	3.05		2.80
Total elephant bone	2.12	550	2.25	1.47	0.15		0.10

By contrast, in the case of synthetic compounds mixtures or of bone, dentine and enamel, the amount of pyrophosphate decreased when the heating time was increased.

4. Influence of grinding on the pyrophosphate formation

When TCPH and CaCO₃ or CaO were mixed and ground in the micromill for 10 min, the pyrophosphate yield was always the same as for pure TCPH: in this case, the degree of intimacy of the two phases, TCPH and CaCO₃ or CaO, was not sufficient to allow penetration of Ca in the phosphate during the thermal treatment.

By contrast, when the two mineral phases are mixed in the colloidal ball mill, the crystals are more finely divided (not measurable) and the mixture becomes more homogeneous so that a reaction in the solid state may occur during the heating period, in such a way that the lattice is completed by calcium and turns into hydroxyapatite (or carbonato-apatite). To obtain this result, grinding must be performed for a rather long time and the pyrophosphate yield decreases when this time increases: for instance, after 20 hr grinding the sample of a mixture (Ca/P weight ratio=2.19) contains 2.75 per cent P as pyrophosphate instead of 12.72 per cent after 1 hr and 10.55 per cent after 4 hr.

The same observation may be made when bone is used: the smaller the bone particles, the less pyrophosphate was found after heating at 400°C for 15 hr. But in this case, the proximity of the two phases present in bone salts cannot be taken into account alone because the organic material is still present during grinding;

presumably, this treatment does not modify the relative positions at the submicroscopic level of the phosphate crystals and the calcium carbonate particles which are embedded in the collagen and the mucopolysaccharides of bone organic material.

5. Influence of organic material removal

When bone and dentine powders are treated (30 min) by the classical Gabriel's method (FABRY, 1959) or by a technique modified by RICHELLE (1960), heated at 300–350°C and dissolved in HCl for the pyrophosphate determinations (N assay negative—BEAULIEU *et al.*, 1950), the pyrophosphate percentage is very low. For instance, a sample of total bone (Ca/P weight ratio=2.18) after heating at 350°C contained 3.42 per cent P as pyrophosphate; if previously treated, this value fell to 0.62 per cent. For dentine the corresponding values were 3.99 per cent (300°C) and 0.42 per cent.

Once more, bone and dentine behave as TCPH and CaCO_3 mixtures: for instance, heated to 400°C, a sample of such a mixture with a Ca/P weight ratio of 2.15 (1 mole TCPH+1 mole CaCO_3) gave 9.44 per cent P as pyrophosphate, but after treatment by KOH-glycerol and washing with water, the yield of pyrophosphate was only 0.95 per cent.

Different results were obtained with elephant enamel (Ca/P=2.12) after heating at 500°C. This material contained 2.14 per cent P as pyrophosphate if not treated by KOH-glycerol and 2.02 per cent if treated. Gabriel's ashing did not influence the yield of pyrophosphates.

It may be concluded that ashing by Gabriel's method induces remodelling of bone and dentine mineral even if the reagents are removed by alcohol washing (RICHELLE, 1959); calcium belonging to the non-phosphoric part of bone and dentine comes into close contact with the apatitic phase during the ashing procedure in such a way that phosphoric acid or pyrophosphate groups may react with it and hydroxyapatite is formed. The same conclusion may be drawn for the mixtures of TCPH and CaCO_3 and this is evidence that the remodelling process really occurs during the treatment by KOH-glycerol.

The ashing of enamel does not change the amount of pyrophosphate groups because keratin is not destroyed during Gabriel's treatment.

DISCUSSION

Pyrophosphate determination provides a means of comparing the behaviour of two compounds with the same Ca/P ratio, one of them having all the calcium ions in the phosphate lattice and the other not.

Bone and dentine behave as TCPH and CaCO_3 or CaO mixtures with the difference that the maximal amount of pyrophosphate is found at a lower temperature than for a mixture of synthetic inorganic compounds. Although we may not extrapolate and affirm that bone and dentine are absolutely similar to these mixtures, we can only deduce that the main constituent of bone and dentine mineral is an apatitic phosphate compound with a Ca/P ratio lower than hydroxyapatite (Ca/P=2.15), the high Ca/P ratio determined by analysis depending on extra calcium which

is not phosphate bound and which is not in close contact with the phosphate phase. Furthermore, we may not assume that the amount of pyrophosphate we found in bone and dentine mineral after heating gives strictly quantitative information regarding the constitution of the main component of these tissues: the non-apatitic calcium may indeed react with the hydrogen bounded acidic phosphate groups ($\text{O}_3\text{PO}-\text{H}-\text{OPO}_3$) or the pyrophosphate groups of the apatitic phase during the heating and the amount of pyrophosphate we found represents a minimal value which, in reality, may be much higher.

It seems likely that the temperature of 325°C , which gives the maximal amount of pyrophosphate, corresponds to the destruction of the organic material of bone and dentine; the latter reaction is exothermic and increases the internal temperature of the particles, even if burning does not occur. We have determined the temperature in the crucible containing total bone powder and found that it reaches 575°C when the oven is at 500°C . Moreover, when TCPH is suspended in gelatin, the maximal theoretical level of pyrophosphate appears at a temperature which is much lower than observed when this compound is ignited alone. A series of control experiments is now in progress in order to establish the best conditions to examine separately three associated processes, namely:

- (a) the thermal dehydration of the acidic phosphate groups of the low calcium apatitic phosphate, inducing the formation of P_2O_7 groups available for the chromatographic partition,
- (b) the destruction of the organic materials, and
- (c) the resultant liberation of the non-apatitic calcium which may react during heating with the apatitic phase to form hydroxyapatite (or carbonato-apatite).

It may be inferred from our results that in bone and dentine the two phases are kept apart by the organic material; the latter is so much more rapidly destroyed by heating that the particles are small and this explains why the amount of pyrophosphate decreases with the bone particles size obtained by grinding.

Consequently, it seems rather difficult to extrapolate from mineral carbonate-hydroxyapatite to bone or dentine as did ENGSTRÖM (1960), who considers that bone mineral could be hydroxyapatite with amorphous calcium carbonate absorbed into it in some way. The presence of the organic material in biological tissues must be taken into account and it certainly plays a part in preserving the structure of the two mineral components. If the organic material is destroyed by ashing, these two phases are able to react and hydroxyapatite may easily be formed.

The behaviour of enamel is different because the amount of organic material present in this tissue is small, consequently liberating less energy during ignition. Furthermore it is more slowly destroyed by heating than the organic phase of bone and dentine.

As a first approximation, it may be assumed that the inorganic fraction of enamel corresponds partly to a phosphocalcic compound which behaves just as TCPH and which is kept apart from extracalcium by heat-resistant keratin. As far as enamel is concerned, the three phenomena occurring concurrently during heating may be partly disconnected.

Acknowledgements—This work was supported by grants of the National Institutes of Health (D-878), United States Public Health Service and of the School of Aviation Medicine through the European Office of the Air Research and Development Command (contract No. AF 61(052)-268).

The authors are indebted to Dr. O. HOLMES who improved the manuscript.

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THE FORMATION OF CALCULUS-LIKE DEPOSITS BY PURE CULTURES OF BACTERIA

W. H. T. BOWEN* and MARION N. GILMOUR

Eastman Dental Dispensary, Rochester, New York, U.S.A.

Abstract—Actinomyces and Leptotrichia formed a calculus-like deposit on single-rooted teeth incubated in a brain-heart infusion—thioglycollate broth. Polarized light, densitometric tracings and microscopic studies confirmed the similarity of the deposit to calculus formed *in vivo*. It is suggested that the role of Actinomyces and Leptotrichia in calculus formation is to enhance the rate of deposition of calcium salts.

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THERE is still uncertainty as to the part bacteria play in the formation of calculus deposits on the teeth and how any such effects are produced. Filamentous organisms, the oral Actinomyces and Leptotrichia (NAESLUND, 1925; BIBBY, 1935; WASSERMAN, MANDEL and LEVY, 1958) have been implicated most frequently, and it has been indicated that they not only provide a mesh-work which entraps precipitating mineral salts, but that they also attach the deposits to the tooth surfaces (BIBBY, 1935; NAESLUND, 1926; ZANDER, 1953). It has also been suggested (BIBBY, 1935; NAESLUND, 1926) that the metabolic activity of bacteria might produce localized environmental conditions conducive to the precipitation of mineral salts.

So far, however, it has not been shown that these organisms do, in fact, play a part in calculus formation. The evidence to support this possibility is circumstantial. It consists of the histological demonstration of large numbers of filamentous bacteria in calculus (NAESLUND, 1925; BIBBY, 1935), their cultivation from it (BIBBY, 1935; NAESLUND, 1926) and the failure of calculus-like deposits to form *in vitro* in saliva in which bacterial growth has been prevented (BIBBY, 1935). The availability of pure cultures of oral Actinomyces and Leptotrichia (GILMOUR and HUNTER, 1958) made it seem desirable to test whether these organisms, either separately or together, would produce calculus-like deposits on extracted teeth and thereby to cast some light on the possible importance of Actinomyces and Leptotrichia in calculus formation.

EXPERIMENTAL PROCEDURE

Single-rooted caries-free teeth were washed with distilled water, cleaned with pumice on a rotary dental brush and autoclaved. A single sterilized tooth was then placed, root downward, in a tube containing 5 ml of a medium (BHI-Thio broth) made by dissolving brain-heart infusion (Difco) 18 g, NIH thioglycollate broth (Difco) 14 g, and yeast extract (B.B.L.) 5 g, in 1000 ml distilled water adjusting to

* Department of Dental Science, Royal College of Surgeons of England, London W.C.2.

pH 7.3, and autoclaving at 15 lb pressure for 15 min. The calcium content (BIEDERMAN and SCHWARZENBACH, 1948) was approximately 0.60 mg/ml and the total phosphate (WILLARD and CENTER's method, 1941) was 0.023 mg/ml. Half way through some tests equal amounts of calcifying solution (SOBEL *et al.*, 1957) and BHI-Thio broth were substituted for the regular medium. The tubes were inoculated with facultative or anaerobic Actinomyces or Leptotrichia, or both, and incubated aerobically at 37°, except for the anaerobic Actinomyces for which pyrogallol-sodium carbonate seals were employed. Tubes inoculated with streptococci or aerobic diphtheroids, isolated from a human mouth, and uninoculated tubes containing the medium and sterile teeth were used as controls. Every second day the medium was replaced with fresh medium and the pH of the used medium from each tube was measured with a Beckman pH meter. In each instance the teeth were examined in their tubes and the "feel" of any deposit determined with a sterile dental instrument. After 2 or 3 weeks, when luxurious bacterial growth had covered the roots of the teeth, the medium was renewed and the teeth examined daily. At this time BHI-Thio broth-calcifying solution mixture was substituted for regular medium in half of the tubes. After a further 3 weeks' incubation the teeth were removed, fixed in 10% formalin, mounted in Ward's bioplastic, sectioned at 900-100 μ thickness and polished, using a carborundum wheel and a fine stone. These sections were studied, using Gram-Weigert stain, polarized light and microradiography. For study under polarized light, sections were immersed in aqueous mixtures of potassium iodide and red mercuric iodide having refractive indices of 1.4 and 1.68 (SONI and BRUDEVOLD, 1959). For microradiography contact films were made using a Picker X-ray unit fitted with a copper Machlett tube at 20 kV and 10 mA and a target film distance of 10 cm. An Eastman Kodak microdensitometer using a slit width of 8 \times 8 μ was used to make densitometric tracings.

FINDINGS

Satisfactory growth occurred in all the experimental tubes, and Gram-stained smears of the medium at the end of the experiments demonstrated the presence of Actinomyces and Leptotrichia. Any tube becoming contaminated could be detected by appearance of turbidity rather than granular growth in the medium. All such tubes were discarded. It is noteworthy that layers of the organisms attached themselves primarily to the cementum surface of the tooth roots and appeared on the crown only in pits in which brown stain had survived the original cleaning procedures. The used media had a pH of 6.2-6.5. As incubation continued, both in the regular and the calcium enriched media, the felt-like network of micro-organisms on the roots of the teeth became tougher and harder to the feel of the dental instrument, and the deposits which formed on the stained areas of the crown increased in diameter. No such formations appeared in the control tubes. At the conclusion of the incubation period the roots were covered with a hard tenacious tan-coloured mass resembling calculus in appearance (Figs. 1 and 2). These deposits had a hardness similar to calculus, but were more brittle.

Microscope studies of sections of deposits (Fig. 3) revealed several points of resemblance to "natural" calculus formations on human teeth. These included the presence of a cuticle-like layer between the tooth and some areas of the deposit and the existence of a layering effect resembling incremental lines. In addition clumps of bacteria could be observed dispersed throughout the deposit, in the Gram-stained sections. Examination under polarized light revealed variable degrees of mineralization with lower levels about the outside and in the region of cracks. The micro-radiographs frequently showed radiopacity extending from the surface to the cementum and rarely a similar distribution of radiolucent patches. Alternating

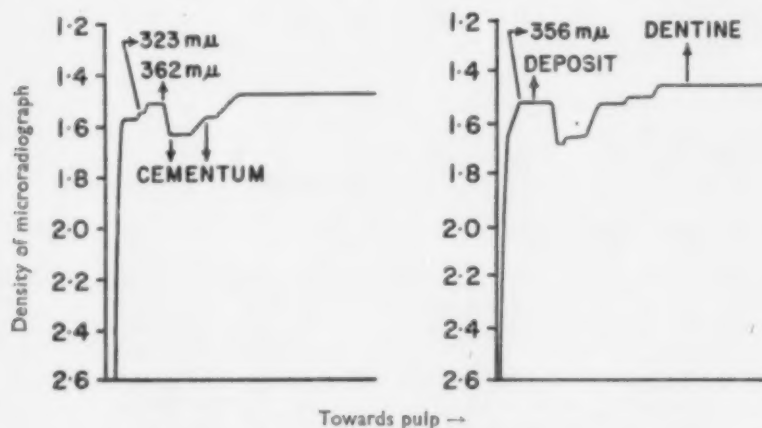


FIG. 4. Densitometric tracings of microradiographs of teeth having deposit.

layers of radiolucency and radiopacity parallel to the surface were common. Densitometric tracings (Fig. 4) across the microradiograph showed that the radiopacity increased from the outside inwards until a plateau was reached with density comparable to that of the dentine.

DISCUSSION

The premise that *Actinomyces* and *Leptotrichia* are of importance in calculus formation is supported by our finding that calculus-like deposits are formed on teeth kept in cultures of these organisms, but not in similar inoculated and uninoculated control tests. That this occurred *in vitro* in a medium with a relatively low calcium concentration and at a pH comparable to that of saliva strengthens the likelihood that these organisms have some special significance in calculus formation *in vivo*. That the product of the organisms is similar to calculus is supported by the gross and histological similarity between the deposits so formed and naturally-occurring human calculus. In the latter respect, it is interesting to note the existence of a layered pattern resembling the incremental lines of natural calculus and the presence of bacterial masses (MANDEL and LEVY, 1957) in the deposits.

The experimental findings viewed in conjunction with the absence of deposits in the controls indicate some relationship between the presence of Actinomyces and Leptotrichia and the deposition of this calculus-like material on the teeth. This may seem difficult to reconcile with the report that calculus-like accretions occur in germ-free rats (FITZGERALD and McDANIEL, 1960). If the deposits observed in their experiments are indeed akin to those that we have produced *in vitro*, it seems possible that the role of these organisms may be no more than to enhance the rate of deposition of calculus.

Acknowledgement—This work was supported in part by the National Institutes of Health, Grant No. D 821, and the Colgate-Palmolive Company.

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THE FORMATION OF CALCULUS-LIKE DEPOSITS BY PURE CULTURES OF BACTERIA



FIG. 1



FIG. 2

FIGS. 1-2. Teeth showing calculus-like deposit on root surfaces. $\times 20$.

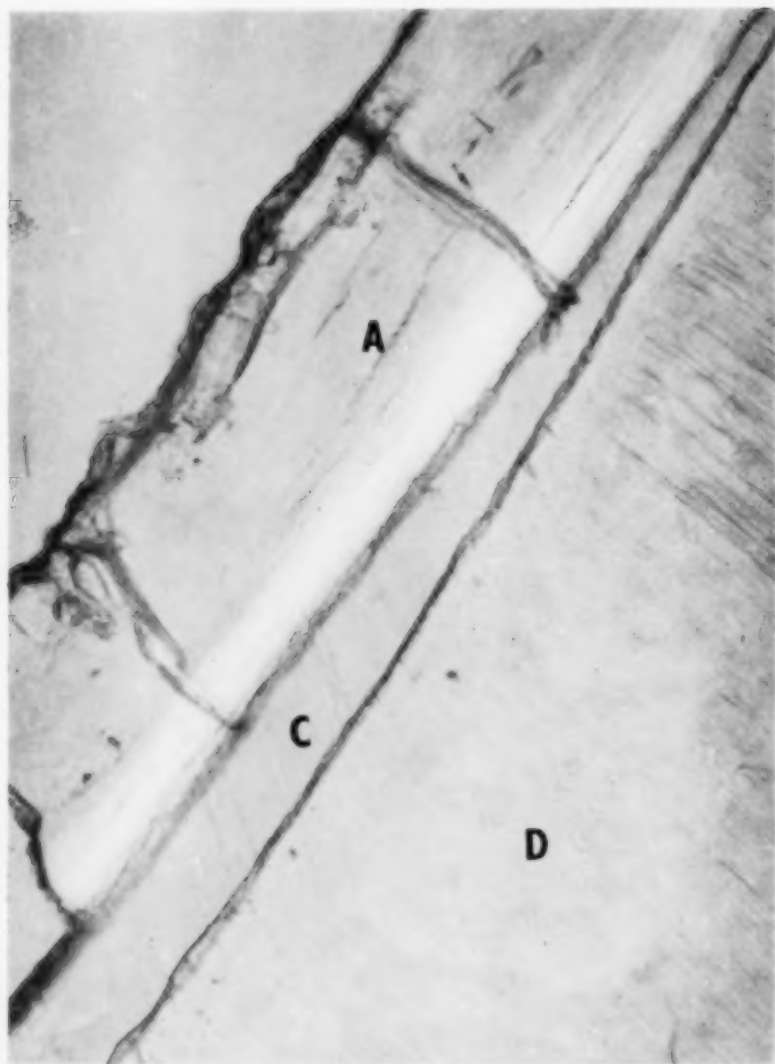


FIG. 3. Unstained ground section with calculus-like deposit (A) in apposition to cementum (C); D, dentine. $\times 150$.

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EFFECTS OF UREA DOSAGE ON UREA CORRELATIONS IN HUMAN PAROTID FLUID AND BLOOD SERUM

I. L. SHANNON and J. R. PRIGMORE

Dental Sciences Group and Medical Sciences Division, School of Aviation
Medicine, U.S.A.F., Brooks Air Force Base, Texas, U.S.A.

Abstract—Forty-four healthy young adult male subjects were given oral doses of urea and parotid fluid and serum urea levels were studied for 1-3 hr. A highly significant correlation between urea in serum and in parotid fluid ($r=0.982$) was found. The indication was that, with flow rate carefully controlled, parotid fluid could be used interchangeably with serum in urea determinations, regardless of the magnitude of the blood concentration.

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UREA nitrogen determinations in blood and urine are of extreme importance in the diagnosis of systemic disease, particularly those disorders affecting protein metabolism and/or renal function. The collection of these fluids at frequent intervals is often impracticable. Thus, if reliable urea determinations could be carried out with a more expeditiously collected fluid, the advantages would be great. It was toward this end, the determination of urea in parotid fluid and the relation of this level to that found in blood serum, that the present study was directed.

MATERIALS AND METHODS

A total of forty-four healthy young (17-22 years) adult males served as volunteer subjects. Participants ate breakfast at 0530 hours, an identical food selection being served to all members of each of the four experimental groups. Control 10 ml venous blood samples and 10 min parotid fluid specimens were collected from each subject between 0730 and 0800 hours. Parotid flow was elicited by having the subjects chew one stick (approximately 3.0 g) of chewing gum (furnished by Dr. Robert Heggie, American Chicle Company) and the fluid was collected in centrifuge tubes graduated at 0.1 ml intervals. Before the test samples were collected, each subject was allowed a 10 min chewing period in which to become familiar with the stimulation procedure and with the presence of the double-walled metal suction device (SHANNON, PRIGMORE and CHAUNCEY, 1961) in place over the orifice of Stensen's duct. Throughout the studies the gum bolus was renewed at 5 min intervals.

Urea nitrogen was determined in both fluids on the Autoanalyzer (Technicon Instruments Corporation, Chauncey, New York). SHANNON and PRIGMORE (1960a,b) have reported the determination of urea nitrogen in parotid fluid diluted 1:5 with 10% trichloroacetic acid. A detailed description of the automatic methods employed with both fluids in the present study is presented by SHANNON and PRIGMORE (1961).

A series of three experiments comprise the present study. In the first phase the relationship between the urea concentration of plasma, serum, and whole blood was

determined. In the second, eleven subjects were administered 30 g of urea orally in cold tomato juice and their serum and parotid fluid urea concentrations were determined over a 1 hr period. An additional ten persons were followed for 3 hr under the same conditions. In the final experiment twelve persons were given 60 g of urea and studied for 1 hr, and eleven subjects, given the same dosage, were followed for 3 hr.

A within subject correlation coefficient between urea concentration in serum and in parotid fluid was calculated for each subject. Tests were carried out to determine whether or not there were differences between correlations due to different concentrations of urea or to the different schedules of sampling.

RESULTS

Performance of method

Duplicate urea determinations were carried out on forty samples of fresh serum with the following urea results: mean, 34.2 mg per cent; standard deviation*, 0.296; coefficient of variation, 0.86 per cent. Comparable figures were found for the parotid fluid procedure (SHANNON and PRIGMORE, 1960a,b).

Whole blood, plasma and serum urea

A blood sample was collected from each of nine subjects both before and 30 min after the ingestion of 30 g of urea. Urea was determined on whole blood, plasma and serum and correlation coefficients between the variables were calculated. These coefficients were as follows: whole blood and plasma, 0.990 before dosage; 0.989 after dosage; whole blood and serum, 0.988 before dosage, 0.978 after dosage; plasma and serum, 0.996 before dosage, 0.990 after dosage. The urea findings for the three fluids were highly correlated both before and after urea was ingested, all of the correlations being essentially the same. It was of interest, however, that the subjects did not maintain the same rank order after dosage as they had previously, that is, the increase in urea 30 min after taking urea orally was not the same for all subjects.

Thirty gram oral dose

After control samples were taken, eleven subjects were given 30 g of urea and sampling was continued for 1 hr. Blood was drawn 20, 40 and 60 min after dosage while parotid fluid was collected continuously, twelve 5 min samples over the 1 hr period. The urea mean prior to dosage was 36.4 (S.D., 4.74) mg per cent while that for parotid fluid was 23.5 (S.D., 4.07) mg per cent. At these sampling intervals the serum concentration reached a maximum after 40 min at 97.6 (S.D., 9.17) while the parotid level reached 75.8 (S.D., 14.31) mg per cent after 35 min. The blood level remained high, 94.6 (S.D., 8.72) mg per cent after 1 hr, while the parotid fluid concentration dropped to 61.6 (S.D., 14.55) mg per cent. These results are plotted in Fig. 1 as percentile deviations from the true control level arbitrarily converted to zero.

$$\text{* S.D.} = \sqrt{\left[\frac{S(d-\bar{d})^2}{N-1} \right]} \quad (\text{Where } d \text{ represents the actual difference between results of duplicate urea determinations on the same serum sample}).$$

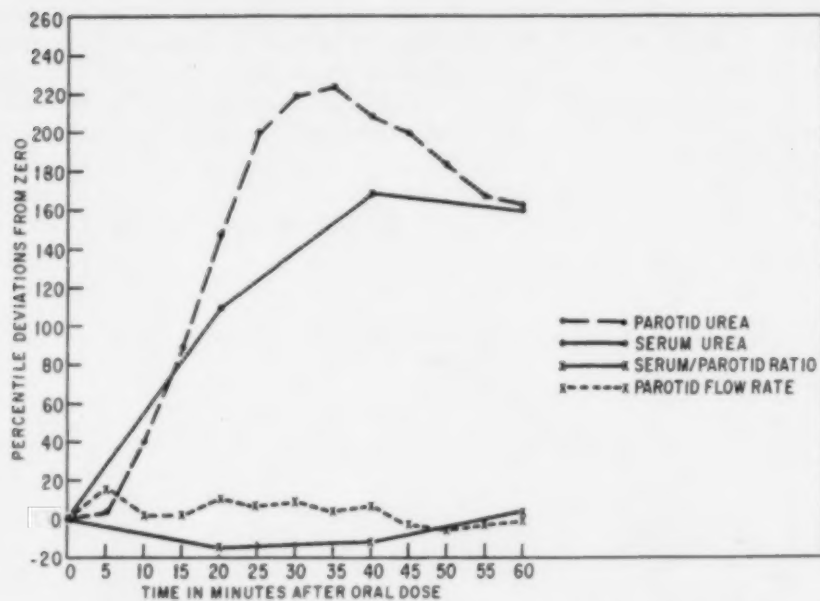


FIG. 1. Serum and parotid fluid results during 1 hr after 30 g urea orally. (Control findings taken as zero).

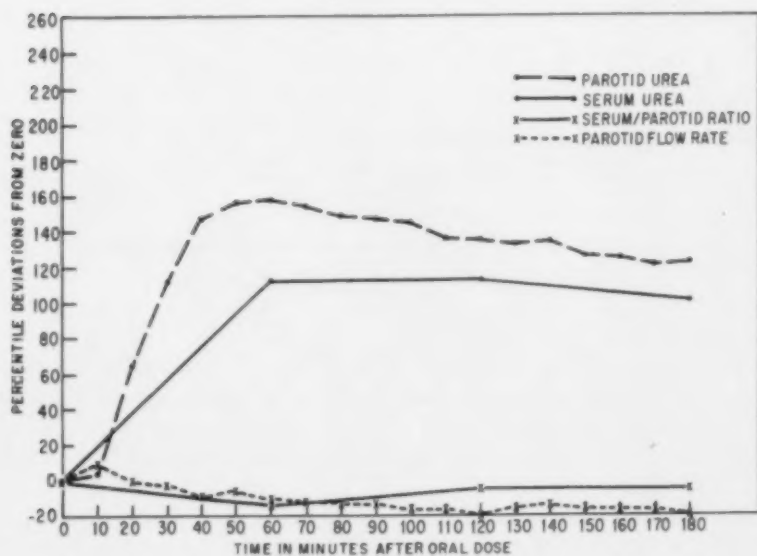


FIG. 2. Serum and parotid fluid results during 3 hr after 30 g urea orally. (Control findings taken as zero).

With the same dosage level ten subjects were studied over a 3 hr period. Blood was taken at hourly intervals and eighteen consecutive 10 min parotid fluid samples were collected after dosage. The control serum urea mean was 36.1 (S.D., 10.91) mg per cent and that for parotid fluid was 22.0 (S.D., 3.22) mg per cent. The serum level increased to 77.0 (S.D., 9.78) after 1 hr, maintained this level exactly at the 2 hr time interval, and decreased to 72.7 (S.D., 5.76) mg per cent at the 3 hr interval. The parotid fluid concentration peaked after 60 min at 56.5 (S.D., 13.27) and fell stepwise to 51.8 (S.D., 13.04) and 48.9 (S.D., 10.70) mg per cent after 2 and 3 hr, respectively (Fig. 2).

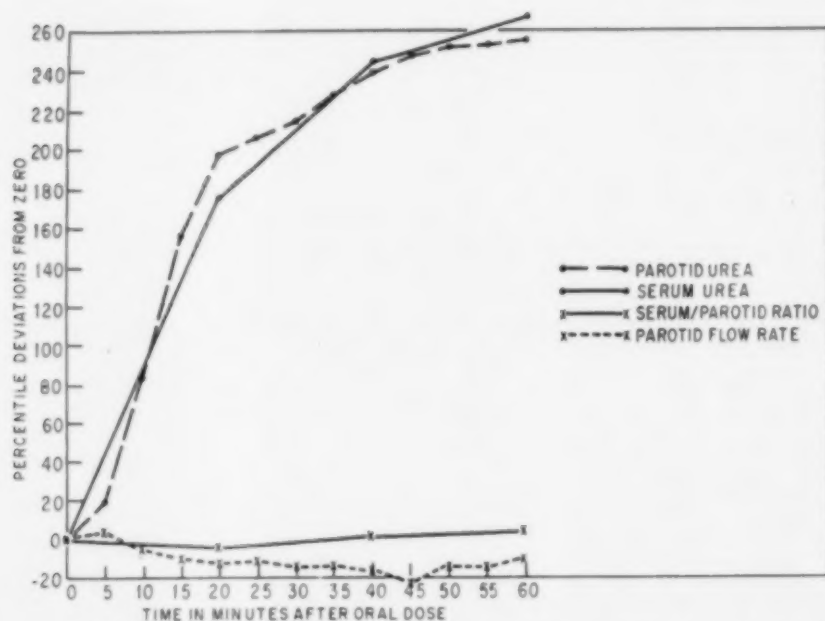


FIG. 3. Serum and parotid fluid results during 1 hr after 60 g urea orally. (Control findings taken as zero).

Sixty gram oral dose

Twelve subjects were given an oral dose of 60 g of urea and were sampled for 1 hr. The control serum urea mean of 29.7 (S.D., 5.71) mg per cent increased throughout the 1 hr period to a final level of 109.2 (S.D., 23.6) mg per cent. The parotid fluid mean increased from a starting level of 23.6 (S.D., 3.62) to a final value of 83.7 (S.D., 19.70) mg per cent (Fig. 3).

Under identical dosage conditions eleven subjects were followed for a 3 hr period. The starting serum urea mean of 31.3 (S.D., 5.58) mg per cent peaked at 118.3 (S.D., 10.37) mg per cent after 2 hr and dropped to 112.3 (S.D., 10.94) 1 hr later. The parotid fluid mean increased from 22.6 (S.D., 3.27) to 81.8 (S.D., 18.86) mg per

cent after 80 min and decreased steadily to 70.4 (S.D., 10.93) mg per cent at the end of the 3 hr period (Fig. 4).

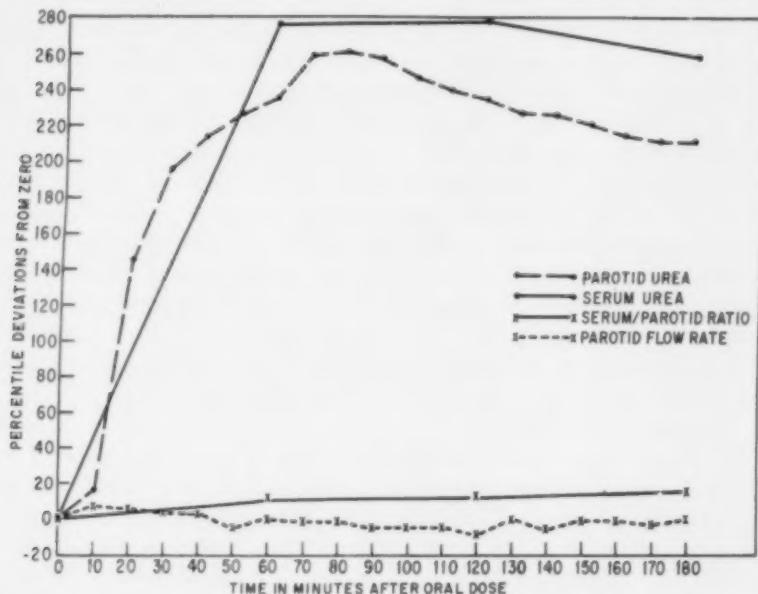


FIG. 4. Serum and parotid fluid results during 3 hr after 60 g urea orally. (Control findings taken as zero).

DISCUSSION

That salivary urea levels might be used clinically in the diagnosis of uremia was noted by HENCH and ALDRICH (1922), who found that the salivary level varied in proportion to blood urea levels. The observations of others (GAD ANDRESEN, 1921; SCHMITZ, 1922; SMITH, 1922; LANDSBERG, 1923; MORRIS and WAY, 1924; CORKHILL, 1925; CALVIN and ISAACS, 1925; BINET, 1926; NIKIFORUK, 1956) lend support to this contention. Salivary urea has been reported as containing 80 (HENCH and ALDRICH, 1922), 89.4 (SCHMITZ, 1922), 76.1 (UPDEGRAFF and LEWIS, 1924), and, with ammonia, 74 (NIKIFORUK *et al.*, 1956) per cent of the blood urea level. It has been reported, however, that the salivary urea index did not correlate well with blood findings in pathologic cases (STEALY 1928), that urea nitrogen could be found in the saliva of only three of ten human subjects (FERRIS, SMITH and GRAVES, 1923), and that urea in saliva signified a pathologic state of kidney function (FERRIS, 1920). Theoretically it would be expected that urea levels in saliva and in blood would be reasonably similar since it has long been known (MARSHALL and DAVIS, 1914) that the urea content of virtually all organs and tissues is approximately uniform and is very similar to that of blood; that when urea is administered it spreads to all parts of the body very quickly; and that the rate of excretion of urea in normal animals is directly proportional to the concentration of urea in the blood.

A pronounced disadvantage in the use of mixed saliva for urea nitrogen determinations is the presence of bacterial contamination and the resultant urease activity. The breakdown of salivary urea by bacterial ureases makes it necessary that both urea and ammonia nitrogen be measured. In this regard, it has been established (SCHMITZ, 1922; BARNETT and BRAMKAMP, 1929; BRAMKAMP, 1937) that the concentration of ammonia in pure parotid secretion was always negligible and that parotid secretion could be incubated at 37°C for 24 hr without demonstrable loss of urea (ALBRECHTSEN and THAYSEN, 1955).

In the present study a highly significant correlation between urea in serum and in parotid fluid was found. For the forty-four subjects receiving oral dosage of urea an average correlation coefficient of 0.982 was calculated. The individual correlations ranged from 0.796 to 0.9997. There were no significant differences between the correlations due to different schedules of sampling or to the greatly varying concentrations of urea in the fluids. It is thus apparent that, within subjects and in values representing means for groups of subjects, the serum/parotid fluid correlation is such that diagnosis from either fluid would seem feasible.

The preponderance of evidence indicates a very definite effect of secretion rate on salivary urea concentration. BARNETT and BRAMKAMP (1929) found a negative correlation between these variables in both whole and parotid saliva. Our previous work in this field (SHANNON and PRIGMORE, 1960c,d) with various forms of stimulation in fifty-two subjects, has provided a significant ($P < 0.01$) negative correlation coefficient of -0.83 for these two variables. MORRIS and JERSEY (1923) voiced the counter-opinion that there was a strong correlation of the positive type between urea concentration and rate of flow. Under paraffin stimulation, flow rate increased by 429 per cent and a like change of 374 per cent was found in urea concentration; acetic acid stimulation increased flow by 560 per cent and urea by 200 per cent; pilocarpine increased flow by 502 per cent and urea by 445 per cent. In all instances the results, incorrectly reported in a paper by CARCO and CANCIULLO (1958), are expressed as urea plus ammonia nitrogen. This positive correlation was also found by VLADESCO and POPESCO (1929) while BRAMKAMP (1937) did not find a significant flow rate influence on salivary urea.

The graphs of the present study include the results for the urea ratio between serum and parotid fluid when the two fluids were collected concurrently. The mean parotid flow rate for all subjects at each time interval is also plotted. These findings seem to support those of ALBRECHTSEN and THAYSEN (1955) in that a ratio (they calculated parotid/plasma urea) of the urea in these fluids seems to be relatively independent of large variations in serum urea levels. The effect of flow rate on this ratio is not made clear in this experiment since an effort was made to stabilize rather than to vary volume output.

Acknowledgement—The authors express appreciation to J. F. MCANEAR, E. D. DINGER, L. CRAWFORD, R. R. HOGUE and R. P. McMILLAN for technical assistance.

The opinions expressed in this paper are those of the authors and are not to be construed as representing official Air Force policy.

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THE VACUOLAR NATURE OF BONE GROUND SUBSTANCE

J. A. YAEGER

Department of Histology, College of Dentistry,
University of Illinois, Chicago, Illinois, U.S.A.

Abstract—The palates of 1 day old mice were fixed by immersion in chilled 95% ethanol and buffered aqueous 2% osmium tetroxide. Some osmium-fixed tissue was stained with 1% phosphotungstic acid (PTA) in 95% ethanol. All tissue was dehydrated in ethanol and embedded in methacrylate. Thin sections mounted on grids were demineralized in 1% PTA in 50% ethanol or in a saturated solution of disodium ethylene diamine tetracetate (EDTA). Sections demineralized in EDTA were stained with aqueous 2% osmium tetroxide.

After demineralization, submicroscopic vacuoles were apparent in bone interfibrillar ground substance. In relatively mature bone the vacuoles were approximately 20 m μ in diameter and their walls approximately 10 m μ regardless of the preparative reagents used. The vacuoles increased in diameter as newly formed bone matured. This change was interpreted as resulting from the removal of collagen from the ground substance during fibril development and growth.

INTRODUCTION

DURING electron microscopy of demineralized sections of bone which had been fixed in alcohol, areas of distinctly vacuolar ground substance were seen between collagen fibres. A two phase morphologic pattern of this kind in connective tissue ground substances has been postulated to explain physiologic and pathologic changes in negative charge density detected in such tissues (JOSEPH, ENGEL and CATCHPOLE, 1952; ENGEL, JOSEPH and CATCHPOLE, 1954) and morphologic manifestations of a two phase system in tendon (BONDAREFF, 1957), endomysium (CHASE, 1959) and osteoid (DURNING, 1958; CHASE, 1960) ground substances have been observed in the electron microscope. The tissues used for the latter studies have all been prepared by freezing and drying, and none of the observations have been confirmed in tissue which has been fixed in fluid while still wet.

This report includes observations which indicate that the vacuolar nature of bone ground substance is not an artifact caused by fixation, demineralization or staining, and that the vacuolar pattern changes during bone formation and maturation.

MATERIALS AND METHODS

The palates of 1 day old mice were dissected free and split sagittally. The palate halves were immediately immersed in chilled, buffered aqueous 1% osmium tetroxide (PALADE, 1952) or 95% ethanol. The tissues remained in these solutions for 30 min at 4°C and an additional 30 min at room temperature. The osmium-fixed tissues were dehydrated in ascending ethanol solutions (50, 75 and 95%). Some of

these tissues were stained in 1% phosphotungstic acid (PTA) in 95% ethanol for 2 hr. All tissues were then dehydrated in 100% ethanol and were infiltrated and embedded in methacrylate using benzoyl peroxide as initiator and polymerized in an oven at 60°C.

Sections (grey or silver in reflected light) were cut with a Porter-Blum microtome using a diamond knife, floated on 15% acetone in distilled water, flattened with xylene vapour (SATIR and PEACHEY, 1958) and mounted on formvar and carbon coated grids. After mounting on grids, sections were demineralized and stained in one of two ways. Some were floated on 1% PTA in 50% ethanol for 10 min (PEASE, 1960) and briefly rinsed in distilled water. Others were floated on a saturated solution of disodium ethylene diamine tetraacetate (EDTA) in distilled water for 10 min, briefly rinsed in distilled water, then floated on a 2% solution of osmium tetroxide in distilled water for 10 min and again briefly rinsed.

Micrographs were prepared in an RCA EMU-3 electron microscope.

OBSERVATIONS

Bone in sections from blocks which had been fixed and stained in alcohol, osmium, and osmium followed by PTA in 95% ethanol appeared nearly filled with electron opaque inorganic particles. These particles were completely removed from sections floated on PTA in 50% ethanol, or EDTA. In such demineralized sections, the collagen fibres and ground substance could be seen in areas which previously appeared filled with inorganic particles.

Vacuoles in the ground substance were first noticed in sections of tissue which had been fixed in 95% ethanol (Fig. 1). After demineralizing and staining these sections with PTA in 50% ethanol, the ground substance between collagen fibrils was seen to consist of relatively electron transparent vacuoles surrounded by more electron opaque walls. In these and most subsequent sections the diameters of the vacuoles ranged approximately from 15 to 35 μ , with the mean diameter near 20 μ . The thickness of the walls of the vacuoles measured approximately 10–15 μ .

To test the possibility that the vacuoles resulted from fixation, demineralization or staining, sections prepared by several alternate procedures were compared. Sections were cut from tissues fixed in aqueous osmium tetroxide and tissues fixed in osmium and subsequently stained with PTA in 95% ethanol. These sections were demineralized and stained with PTA in 50% ethanol. Vacuoles in these sections were distributed in the same pattern and with the same dimensions as in the ethanol fixed material (Figs. 2 and 6). Osmium fixation alone did not demonstrate the vacuoles as distinctly as alcohol fixation or a combination of osmium fixation and staining with PTA in 95% ethanol. Finally, sections from tissues fixed by each of the three procedures were demineralized in EDTA and stained with aqueous osmium tetroxide. Again, the vacuoles were present, and their dimensions were unchanged (Fig. 3). The osmium staining did not produce as much opacity in the vacuolar walls or collagen fibrils as did PTA.

One day old mouse palate contains many areas of bone formation. In these areas changes in the vacuolar pattern could be observed during the development of

bone matrix. Where bone matrix is differentiating from mesenchyme as the initial phase of endomembranous bone formation, its ground substance was finely granular and contained no vacuoles (Fig. 4). Further into the bodies of the spicules there were areas of transition between granular and vacuolar ground substances (Fig. 5). In the predominantly granular regions (G, Fig. 5) occasional vacuoles and groups of vacuoles could be vaguely recognized. The vacuoles measured approximately $10\text{ m}\mu$ in diameter, and the vacuolar walls approximately $15\text{ m}\mu$ thick. In predominantly vacuolar regions (V, Fig. 5) the vacuoles had approximately the same dimensions as those seen in sections of more mature bone, i.e. the vacuoles were substantially larger ($20\text{ m}\mu$) and the walls slightly thinner (nearer $10\text{ m}\mu$).

In areas in which osteoblasts were forming matrix by apposition upon pre-existing bone surfaces, a somewhat different pattern was seen (Fig. 6). In the zone nearest the surface (left, Fig. 6) the vacuoles were relatively small (approximately $15\text{ m}\mu$ in diameter) and their walls thick (approximately $15\text{ m}\mu$ or thicker). Further into the bodies of the spicules (right, Fig. 6) the vacuoles were larger and their walls thinner, their dimensions equalling those of the more mature matrix.

DISCUSSION

The sensitivity of the walls of the ground substance vacuoles to a number of drugs, hormones, diseases and physiologic states (GERSH and CATCHPOLE, 1960) suggests that the vacuolar pattern of most connective tissue ground substances might be too labile for preservation by the usual fluid fixatives. This might explain their absence from the myriad of published electron micrographs of fluid-fixed connective tissues. Since the negative charge density of bone is higher than that of any other connective tissue (ENGEL *et al.*, 1954; JOSEPH, ENGEL and CATCHPOLE, 1954), the vacuoles of bone ground substance should be small and their walls thick. Comparison of the reported vacuolar dimensions confirm this supposition. BONDAREFF (1957) found the vacuoles in frozen-dried rat tendon to be $200\text{ m}\mu$ in diameter in 16 day foetuses and $100\text{ m}\mu$ in diameter in 2 day old animals. CHASE (1959) has reported the diameters of vacuoles in the endomysium of young mouse diaphragm as ranging from 60 to $120\text{ m}\mu$. The vacuoles in the most mature bone found in the 1 day old mouse palate described above measured approximately $20\text{ m}\mu$ in diameter. The thicker walls of bone ground substance vacuoles apparently facilitated the preservation of ground substance morphology during fluid fixation of wet tissue.

Demineralized bone has been examined previously, and the electron micrographs of this material revealed empty spaces or a finely granular material between the collagen fibres (ROBINSON and WATSON, 1955). The bone had been prepared by demineralization in EDTA followed by osmium fixation and embedding. When mouse palate was prepared in the same way, most of its ground substance was extracted. Apparently the meshwork of inorganic particles in young bone preserved the morphology of its ground substance during fixation, embedding and sectioning. When thin sections were then demineralized, the time of exposure to EDTA was short enough to prevent extraction of the ground substance, or alternatively, the

simultaneous demineralization and fixation by PTA preserved the vacuolar pattern nearly intact.

During bone formation a transition between mesenchyme or osteoid ground substance containing large vacuoles with thin walls and the more dense ground substance of bone containing smaller vacuoles and thicker walls seems appropriate, while the opposite was in fact observed. Since water content decreases as bone matures (NEUMAN and NEUMAN, 1958), and in the absence of convincing evidence for a decrease of polysaccharide-protein complexes, this apparent contradiction can be perhaps best resolved by a consideration of the formation and growth of collagen fibrils. The molecular collagen secreted by osteoblasts and pre-osteoblasts is partly aggregated to form fibrils, but mostly held in the ground substance in a less aggregated form. It is from this pool of less aggregated collagen in the ground substance that material is withdrawn (JACKSON, 1956) during fibril growth as bone matures (ROBINSON and WATSON, 1955).

In areas of its initial differentiation from mesenchyme the ground substance of bone matrix contains an abundance of less aggregated collagen to allow subsequent growth of the rather sparsely distributed collagen fibrils. This ground substance is therefore nearly homogeneous (Fig. 4). As collagen fibrils increase in number and diameter, collagen is withdrawn from the ground substance, and vacuoles of relatively water-rich, colloid-poor material appear. In these areas the vacuoles are small and their walls thick (G, Fig. 5). As the fibrils continue to grow the vacuoles enlarge and their walls become thinner, until an equilibrium is reached (V, Fig. 5).

During the more orderly apposition of matrix upon pre-existing bone, the phase of homogeneous or granular ground substance is minimized. More of the collagen is apparently aggregated into fibrils soon after it is secreted, and the ground substance is vacuolar near the surface of the bone (left, Fig. 6). Again, as the collagen fibrils become more numerous and increase in size, the ground substance vacuoles become larger (right, Fig. 6). The bone described here is immature, but the matrix in sections which were not demineralized was nearly filled with inorganic particles. Under these circumstances additional fibril growth is unlikely, and the vacuolar ground substance probably persists in the oldest immature bone.

Acknowledgements—It is a pleasure to acknowledge my indebtedness to Mrs. ALINA SKRUPSKELIS for technical assistance, and to Dr. JOHN MARBARGER, Miss IRENA KAIRYS and Mr. WILLIAM KADETZ of the University of Illinois Aeromedical Laboratory for providing and maintaining the electron microscopes used in this study.

This investigation was supported by a P.H.S. Research Grant (A-3220) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

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PLATE I

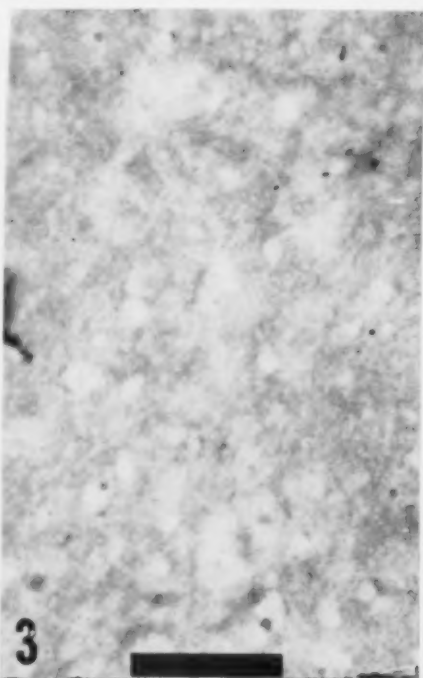
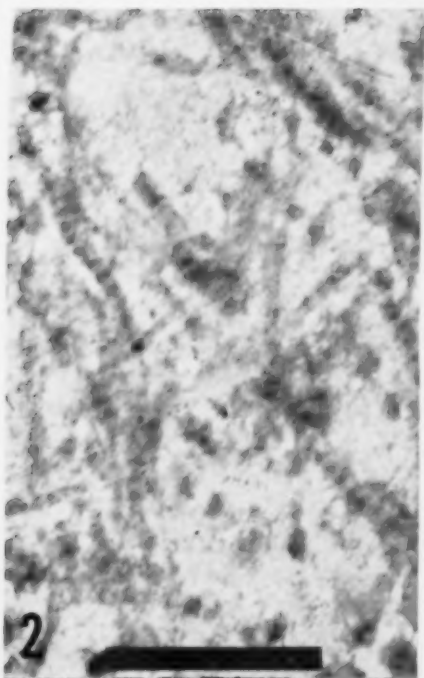
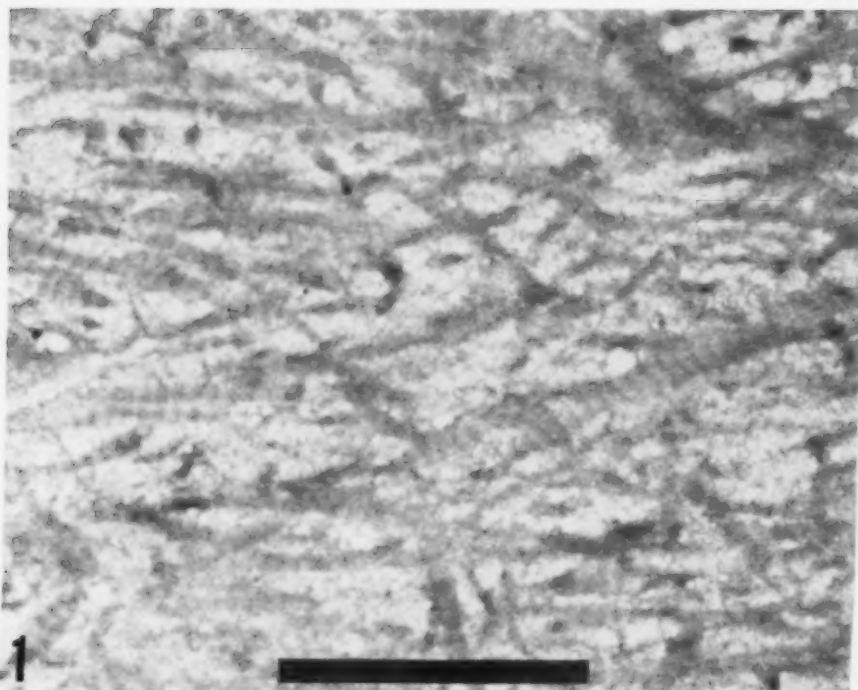
All micrographs are of sections of 1 day old mouse palate. The bars at the bottom of the prints represent one micron.

FIG. 1. Tissue fixed in ethanol, section demineralized and stained with PTA. Collagen fibres and the vacuolar nature of the interfibrillar ground substance are evident. $\times 44,000$.

FIG. 2. Tissue fixed in aqueous osmium tetroxide followed by PTA, section demineralized and stained with PTA. Collagen fibres and vacuolar ground substance are again evident. $\times 30,000$.

FIG. 3. Tissue fixed in ethanol, section demineralized in EDTA and stained with aqueous osmium tetroxide. Electron opacity of collagen fibres is less than in Figs. 1 and 2, while the vacuolar nature of the ground substance is still evident. $\times 21,000$.

THE VACUOLAR NATURE OF BONE GROUND SUBSTANCE



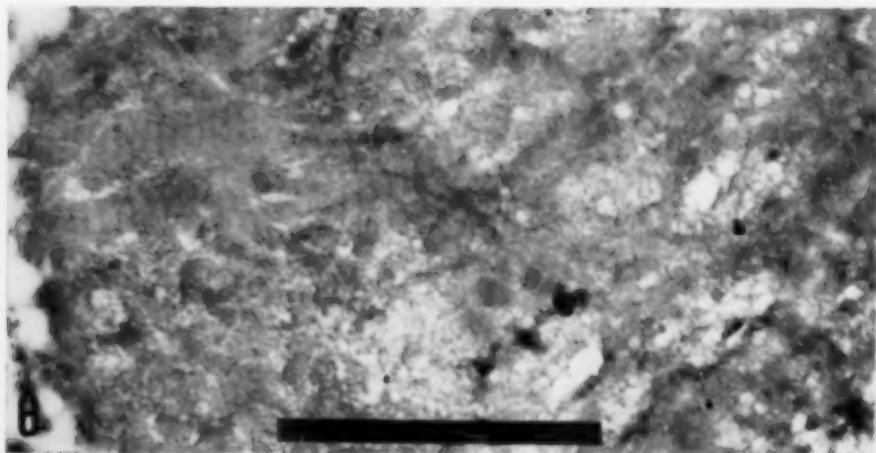
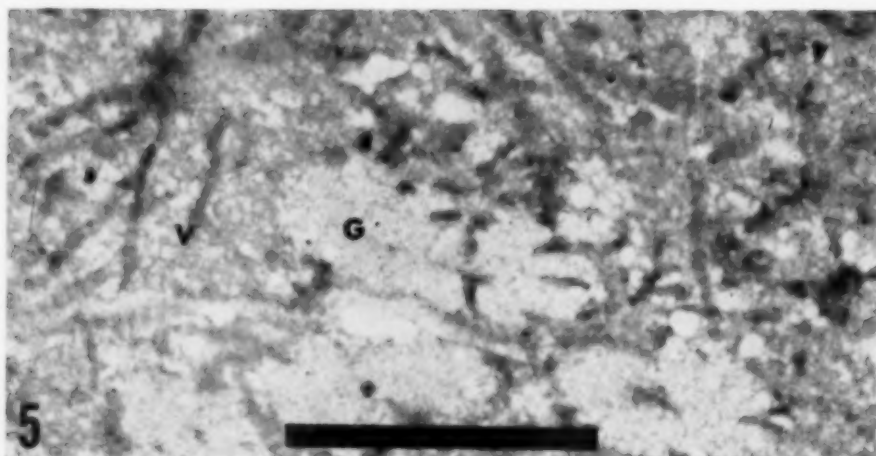
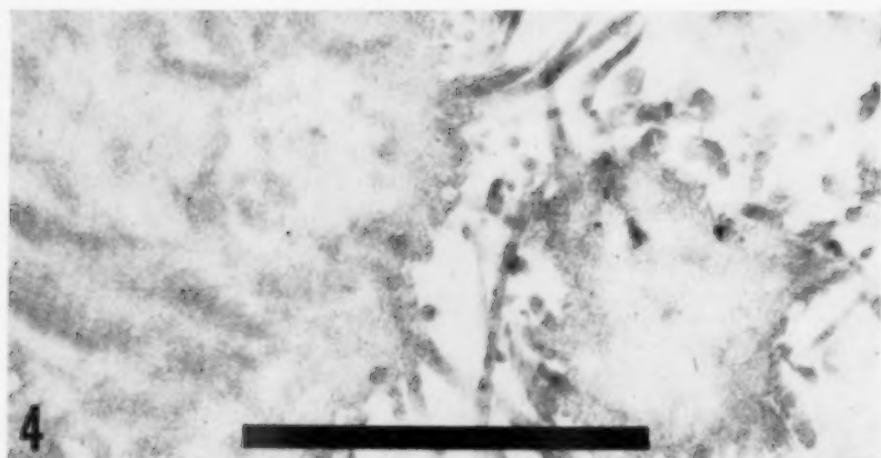


PLATE 2

All micrographs are of sections of 1 day old mouse palate. The bars at the bottom of the prints represent one micron.

FIG. 4. Tissue fixed in aqueous osmium tetroxide, section demineralized and stained with PTA. The finely granular nature of the ground substance in recently differentiated bone matrix is evident. $\times 57,500$.

FIG. 5. Tissue fixed in ethanol, section demineralized and stained with PTA. Transition zone between predominantly granular, recently differentiated ground substance (G) and vacuolar, more mature ground substance (V). $\times 43,500$.

FIG. 6. Tissue fixed in aqueous osmium tetroxide, section demineralized and stained with PTA. At the left is a clear area previously occupied by osteoblast cytoplasm. The vacuoles in the most recently formed matrix (left) are smaller than in the more mature matrix (right). $\times 45,000$.

MUCOUS-CELL METAPLASIA IN HUMAN GINGIVAL EPITHELIUM AND ITS RELATION TO CERTAIN MUCOUS SECRETING TUMOURS

J. J. HODSON

Department of Dental Pathology, University of Sheffield, England

Abstract—Mucous-cell metaplasia of non-neoplastic skin and mucous membrane has not hitherto been recorded. Six cases of such metaplasia are described in cystic epithelial bodies in human gingiva and apparently of surface origin. The significance of this occurrence is discussed in relation to similar metaplasia in odontogenic cysts and certain mucus-secreting tumours.

INTRODUCTION

THE TRANSFORMATION of cell type in the tissues of the body is well known in pathological material, especially neoplasms. WILLIS (1958) gives a comprehensive account of metaplasia in various tissues and organs. Epithelial metaplasia may occur in three ways, squamous or epidermoid transformation of glandular epithelium, glandular transformation of non-glandular epithelium, and the transformation of one type of glandular epithelium into another. WILLIS (1958) notes that glandular metaplasia in non-neoplastic epidermis has not been observed although it does occur in dental cysts as mucous-cell metaplasia of squamous epithelium (HODSON, 1956) which he considers is closely akin to epidermis. However, MONTAGNA (1956) has described sebaceous gland metaplasia in the cells of the skin in hamsters, rabbits and man when unduly irritated and also in the epithelium of the uterine cervix. MONTAGNA suggests that such occurrences show that the cells or the germ layers are not as specific as once believed. It is probable that the development of new sebaceous glands in areas of the cheek mucosa previously devoid of them, described by MILES (1958), is a metaplastic change.

LENNOX and PEARSE (1954) state categorically that "under no circumstances does the epidermis or any of the tumours derived directly from the epidermis produce mucin".

In the present paper six cases are described in which mucous-cell metaplasia had occurred in the gingival epithelium. This type of glandular metaplasia appears not to have been previously observed either in the epithelium of mucous membranes or skin. It may be noted, however, that FELL and MELLANBY (1953) describe the metaplastic change of embryonic cheek ectoderm to mucus-secreting epithelium in tissue culture media containing excess vitamin A.

MATERIALS AND METHODS

Routine examination of the labial interdental papillae of five specimens of gingivectomy tissue from patients with chronic hyperplastic gingivitis showed mucous-cell metaplasia. Three were males aged 14, 24 and 25 years, and two were females aged 17 and 36 years. A sixth case was found in the gum of the mandible of a male who came to autopsy at 80 years. Four of the cases were from the mandible and two from the maxilla. Routine histological examination was carried out and sections stained with haematoxylin and eosin and mucicarmine.

HISTOLOGY

All the five gingivectomy specimens showed the usual features of chronic hyperplastic gingivitis. These consisted of hyperplastic epithelium with varying degrees of parakeratosis, hydropic change and small amounts of acute and chronic inflammatory cell infiltration. The underlying hyperplastic fibrous connective tissue showed oedema and chronic inflammatory cell infiltration, consisting of plasma cells with some lymphocytes, in varying amounts. The striking feature about these cases was that they all showed roughly spherical epithelial bodies close to the epithelium of the free gingiva. In some cases the epithelial bodies could be traced to a connexion with the surface by fine strands of epithelium two or three cells thick; in other cases the spherical bodies were so close to the basal layer that it was not possible to say whether they were part of the surface epithelium, or were merely compressing the basal cells.

In the centre of these epithelial masses varying degrees of early cystic degeneration were present consisting of ballooning of the cells, hydropic change (chiefly intracellular), cell necrosis with pyknosis and nuclear fragmentation. In preparations stained with haematoxylin and eosin, blue staining mucin-like material was present in the areas of cystic breakdown and within enlarged cells in the centres of the masses. With mucicarmine, this material gave the brilliant red stain characteristic of mucin.

In three cases there was only a small amount of mucin present, mostly dispersed amongst the cellular debris in the centre of the epithelial clumps. This breakdown was due to hydropic change in the central cells similar to that shown in Fig. 1. In one of these cases, the centre of the mass was entirely cystic and in addition to a small amount of mucin in the cyst cavity, mucin was present in one or two of the lining cells. In another case, two separate adjacent spherical epithelial bodies were present with a small amount of mucin in one of the clumps.

Fig. 1 shows the fourth case, which was the only one with acute inflammatory cell infiltration, although all six specimens were associated with a chronically inflamed mucosa. In the fifth specimen, there was a number of small subsidiary epithelial clumps adjacent to the largest mass containing the mucous cells (Figs. 2 and 3). In this case larger cystic spaces were present and around these, well-defined mucous cells remained. Mucous material and degenerate cells were present within the cystic spaces.

The sixth case was found in severely inflamed hyperplastic gingiva which had grown over the surface of a retained root of a lower central incisor. Unconnected

with the surface was a periapical cyst. Fig. 4 shows a low power view of two small gingival polyps overlying the carious root. One of these shows an epithelial body just below the surface epithelium. With the mucicarmine stain the central area was strongly positive (Fig. 5). In Fig. 6, an enlarged view of a section stained with haematoxylin and eosin shows more clearly that the centre of the mass is replaced by large, typical goblet cells. A little hydropic change and cell breakdown are also present.

DISCUSSION

It may be only coincidence that, in all six of the cases of mucous-cell metaplasia in the gingiva so far observed, this change should take place in epithelial bodies below the surface showing early cystic changes. The only other metaplasia of this type which has been reported in non-neoplastic lesions occurs in the epithelium of odontogenic cysts (HODSON, 1956; GORLIN, 1957). Besides the mucous cells lining the cysts, some cases showed mucous-cell metaplasia in some of the deeply placed hyperplastic masses in the cyst wall (HODSON, 1956). These are quite comparable to the cases described here in the gingiva. RITCHEY and ORBAN (1953), HODSON (1954) and BHASKAR and LASKIN (1955) have described gingival cysts in the adult, but in none of their cases did they observe mucous cells.

In neoplastic lesions of the mouth mucous cell change is found in muco-epidermoid salivary tumours (STEWART, FOOTE and BECKER, 1945; BHASKAR and WEINMANN, 1955), and in some adamantinomas (HODSON, 1957).

Two other areas in the oral cavity where mucous cells may be found must be mentioned since they may be mistaken for metaplastic change. The first is the occasional occurrence of mucus secreting cysts of the palatine papilla which may open into the mouth. These are derived from remnants of the nasopalatine duct and the cysts formed may be lined in places by ciliated pseudo-stratified columnar epithelium with goblet cells. The other site is in hyperplasia of the oral mucosa associated with denture irritation. On the labial aspect of the alveolar bone this hyperplastic mass may involve the accessory mucous glands and ducts of the lip. In some cases the walls of a terminal duct may be involved in the connective tissue hyperplasia so that the opening on the surface is greatly expanded. In this area the surface of the hyperplastic mass may be covered by stratified squamous epithelium containing mucous cells which is, in fact, terminal duct epithelium. Fig. 7 shows the development of such an area and Fig. 8, an area from the same case with no obvious ductal relationship. There is usually a great increase in the number of mucous cells (Fig. 8). Serial sections are sometimes necessary to show that this is the true origin of the mucus-secreting epithelium. In my view, the mucous-secreting epithelium is hyperplastic rather than metaplastic.

STEWART *et al.* (1945) observe that mucous cells are very sparse in normal salivary gland ducts and that "their presence in muco-epidermoid tumours may be considered to be a metaplastic change". They also state, however, that mucous cells once formed have considerable powers of proliferation. In inflammatory lesions involving mucous-gland ducts in the oral cavity, considerable numbers of mucous cells may be found

in the duct epithelium, and like the case referred to above, I think that these represent a hyperplasia rather than a true metaplasia.

In the case of the mucous cells in the microcyst formations described here, we are dealing with a true metaplastic change in a tissue not normally containing mucous cells. Here, as was postulated in the case of odontogenic cysts (HODSON, 1956), the metaplastic change is thought to be due to a change in cell chemistry brought about by local conditions rather than an atavistic change, or heteroplasia. The formation of keratin and mucin in the same tissue, as in muco-epidermoid tumours and odontogenic cysts, suggests a change of cell metabolism possibly related to sulphur metabolism as suggested by FELL and MELLANBY (1953). The development of histologically perfect goblet cells, particularly well shown in Fig. 6, does not suggest a primarily degenerative condition.

Of numerous gingival microcysts examined some clearly originated from down-growths of the surface epithelium, whilst others either could not be traced to a direct connexion with the surface or appeared to be compressing the basal cells rather than joining them. The evidence suggests that these formations are most probably of surface origin. However, in view of the fact that periodontal epithelial debris and remnants of the dental lamina may remain in the gingival tissue, the possibility that some of these cysts may be odontogenic in origin should be borne in mind. Even in edentulous jaw mucosa, numerous remains of the sheath of Hertwig may be found (HODSON, 1961). If some of these microcyst formations were derived from odontogenic remnants, then the present cases described would fall into line with the mucous-cell metaplasia found in odontogenic cysts. It is not understood why mucous-cell change should be so common (22 per cent) in the epithelium of odontogenic cysts, which primarily arose from the oral epithelium, and yet with the exception of the cases described here be unknown in the surface epithelium of mucous membranes or skin. However, there appears to be no reason why mucous-cell metaplasia should not occur in the oral or other epithelium if conditions are suitable for the necessary biochemical changes.

In view of the similarity of the gingival epithelium to the skin, mention must be made of the observations of LENNOX, PEARSE and RICHARDS (1952), who attempt to postulate an homologous relationship between certain salivary gland type tumours of the skin and sweat glands, and the sweat gland origin of extra-mammary Paget cells (LENNOX and PEARSE, 1954) on the basis of mucin production. The occurrence of mucous-cell metaplasia in the epithelium of a mucous membrane opens the possibility that, given the necessary conditions for neoplasia, a mucous secreting tumour could arise direct from an epithelium which does not normally contain mucin-producing cells; for instance, a muco-epidermoid carcinoma could arise from surface epithelium and not only from salivary gland ducts as hitherto believed. The points raised may be somewhat tenuous but the question of the specificity of types of tumour growth and the problems of their origin are important subjects in oncology. Although LENNOX and PEARSE (1954) contend that mucin is never produced by the epithelium of the skin, the cases described in the present paper show that it can be produced in the epithelium of a mucous membrane.

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PLATE I

FIG. 1. Chronic gingivitis, anterior gingiva, maxilla—male, age 14. Section shows epithelial mass with hydropic change, acute inflammatory cell infiltration and five mucous cells (arrowed) and some mucous strands in fluid spaces. Haematoxylin and mucicarmine. $\times 135$.

FIG. 2. Chronic gingivitis, anterior gingiva, maxilla—female, age 17. Section shows circular hyperplastic masses with mucous cells and cystic changes in A. Haematoxylin and mucicarmine. $\times 170$.

FIG. 3. Higher power of epithelial cystic body at A in Fig. 2. Four mucous cells are shown with mucous strands in the cystic spaces. The arrow shows a small focus of calcification. $\times 350$.

FIG. 4. Two chronically inflamed masses of hyperplastic gingiva covering a mandibular incisor root—male, age 80. An epithelial body is arrowed. Haematoxylin and eosin. $\times 17$.

MUCOUS-CELL METAPLASIA IN HUMAN GINGIVAL EPITHELIUM

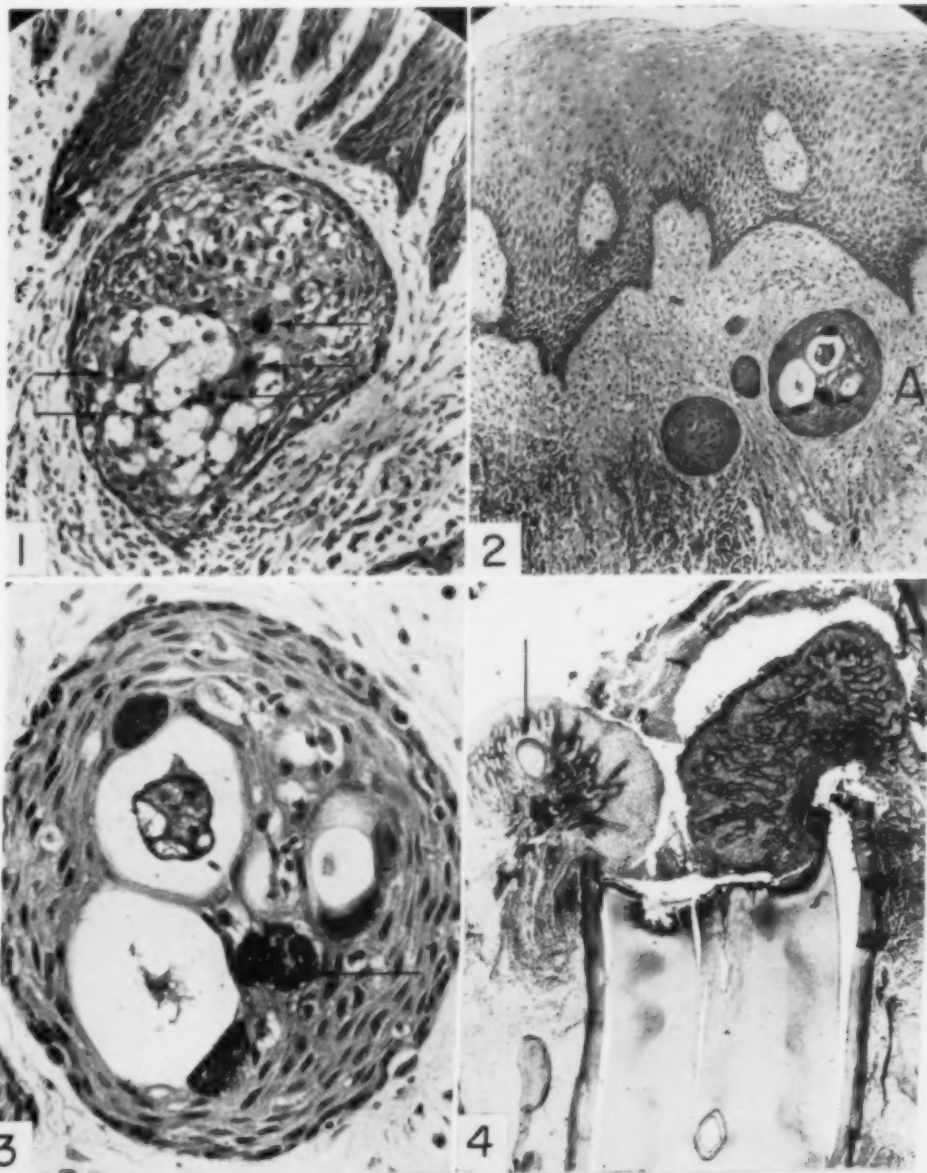
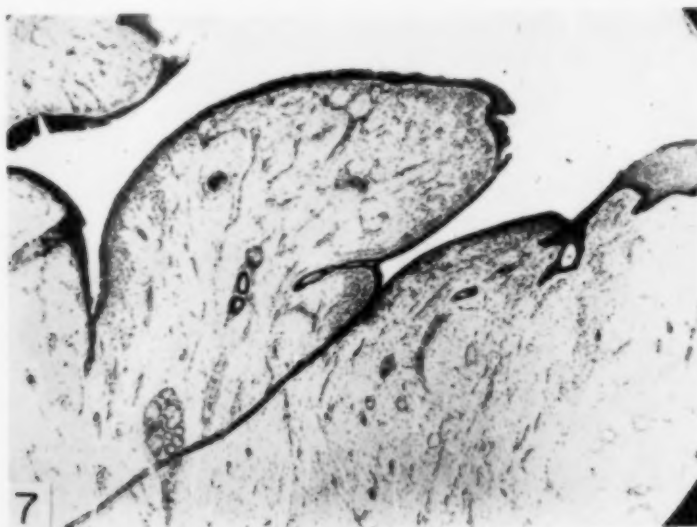
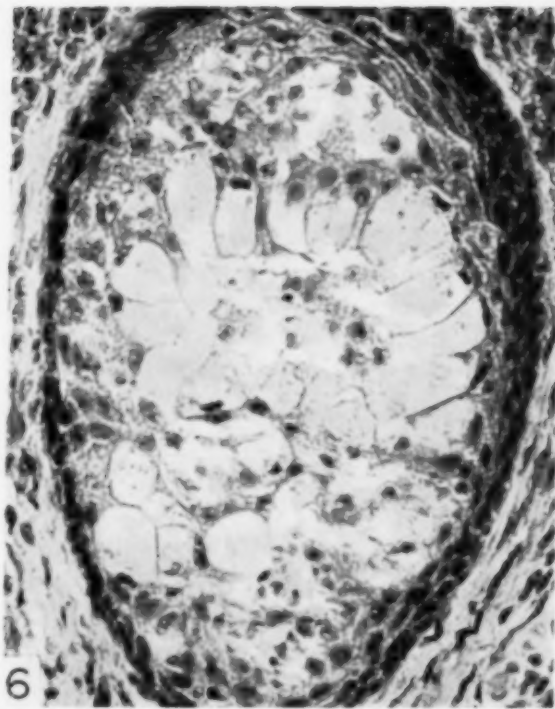


PLATE I



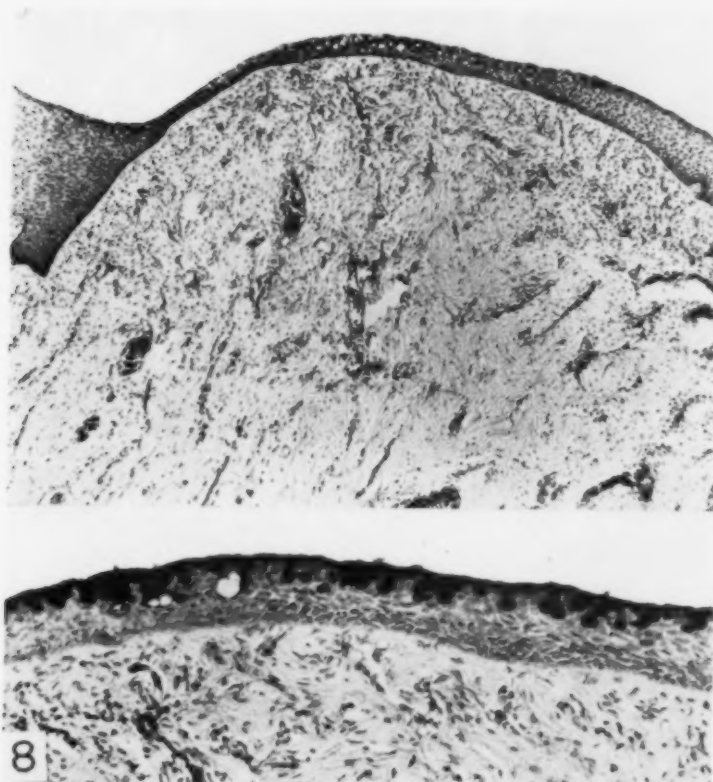


FIG. 8. Section (top) showing surface of another area of hyperplastic oral mucosa from the same case as Fig. 7, which contained numerous mucous cells in the squamous epithelium. In this area no relation to mucous gland ducts was obvious. Haematoxylin and eosin. $\times 46$. The lower photomicrograph shows a higher power view of the mucous cells. Haematoxylin and mucicarmine. $\times 142$.

PLATE 2

FIG. 5. Higher magnification of epithelial body in Fig. 4 showing mucicarmine positive material in the central cells. Haematoxylin and mucicarmine. $\times 150$.

FIG. 6. High magnification showing well-defined goblet cells. Haematoxylin and eosin. $\times 300$.

FIG. 7. Section of area of hyperplastic oral mucosa involving mucous gland ducts, showing the development of a lobe covered by mucous secreting ductal epithelium. Haematoxylin and eosin. $\times 36$.

Vol.
5
1961

REACTION *IN VITRO* OF SOUND DENTINE WITH GLUCOSE, GLUCOSAMINE AND CARBOHYDRATE FERMENTATION AND DEGRADATION PRODUCTS

W. G. ARMSTRONG

Institute of Dental Surgery (University of London),
Eastman Dental Hospital, London W.C.1, England

Abstract—An investigation was undertaken to determine if the organic matrix of sound dentine would react with carbohydrate and related substances. It was found that sound dentine reacted *in vitro* under specified conditions with glucose, glucosamine, the fermentation intermediates glyceraldehyde and dihydroxyacetone, and the carbohydrate degradation products methylfurfural and hydroxymethylfurfural. The dentine products obtained exhibited to a varying degree (i) a browning of the matrix, (ii) resistance to collagenase attack, (iii) reduced concentrations of basic amino acids in acid hydrolysates, and (iv) the presence of material giving carbohydrate reactions, features which are also characteristic of carious dentine preparations. The possible significance of these *in vitro* observations is discussed.

INTRODUCTION

IN A PREVIOUS communication it was reported that the organic matrix of carious dentine contained appreciable quantities of material giving characteristic carbohydrate reactions (ARMSTRONG, 1960). Using anthrone and cysteine methods for carbohydrate analysis it was found that carious dentine contained approximately 4 per cent carbohydrate material. It was also shown (ARMSTRONG, 1961) that acid hydrolysates of carious dentine preparations were characterized by reduced concentrations of certain imino and basic amino acids. These carious dentine acid hydrolysates contained large amounts of humin (ARMSTRONG, 1960).

Other properties of carious dentine are its partial resistance to collagenase attack (PROPHET and ATKINSON, 1953; ARMSTRONG, 1958), and the presence of a fraction apparently derived from collagen, yet completely resistant to collagenase attack (ARMSTRONG, 1960, 1961). This completely collagenase resistant fraction of carious dentine was characterized by a relatively high carbohydrate content of about 12 per cent.

There were theoretical reasons for considering that the presence of carbohydrate in these carious dentine preparations might not be merely a fortuitous association, but that its presence was possibly related to certain of the other phenomena associated with carious dentine. For example, in a series of papers DREIZEN and his co-workers (1949-1958) demonstrated that demineralized sound dentine reacted *in vitro* with certain fermentation intermediates and carbohydrate degradation products to give a brown-coloured matrix. It was suggested that the characteristic browning of the matrix, commonly associated with the carious process, might be accounted for by reactions of this type.

Further, GOTTSCHALK and PARTRIDGE (1950) showed that interaction between glucose and lysine under specific conditions gave rise to brown-coloured products which, on acid hydrolysis, rapidly formed humin products. An extensive investigation into the reaction between sugars and proteins has been reported in a series of papers by LEA and his co-workers (1949-1952). Characteristic features of such protein-sugar reactions included browning of the protein and involvement of the ϵ -NH₂ groups deriving from the lysine residues in the protein. It was also thought that the guanidyl groups of arginine were similarly involved. Acid hydrolysates of such sugar-protein compounds showed reduced concentrations of lysine, arginine and histidine.

Since certain properties of these products were similar to those found in carious dentine (ARMSTRONG, 1959, 1961), the possibility was considered that various features of carious dentine might be explained in terms of reactions occurring between the dentine matrix and carbohydrate material during the carious process. In order to test this theoretical possibility an investigation was undertaken to establish whether reaction between the organic matrix of sound dentine and carbohydrate substances and derivatives could occur *in vitro*, and whether the resultant products showed properties similar to those exhibited by carious dentine. In addition, a preparation of demineralized sound dentine was hydrolysed in the presence of 50 per cent of its own weight of sucrose to determine whether the presence of extraneous carbohydrate had any destructive effect on the imino and basic amino acids during hydrolysis.

MATERIAL AND METHODS

Sound dentine. Sound dentine particles passing a 120 mesh sieve were prepared as previously described (ARMSTRONG, 1958), but the disintegration was carried out using a rapidly oscillating ball mill (Glen Creston micro-disintegrator).

Demineralization of sound dentine particles. The calcified dentine particles were demineralized with 20% ethylene diamine tetra acetic acid (EDTA) as described previously (ARMSTRONG, 1958).

Treatment of sound dentine with glucose. Experiments were carried out on mineralized and demineralized sound dentine. The procedure adopted was similar to that used by LEA and HANNAN (1949) in their studies on the reaction between casein and glucose. 50 mg samples of demineralized sound dentine particles were weighed into 5 ml ampoules. With mineralized dentine particles 200 mg quantities were used. A 30% solution of D(+) glucose (AnalaR grade) was prepared, and the pH brought to 8.5 by the addition of sodium bicarbonate. 0.25 ml aliquots were added to each ampoule and thoroughly mixed with the dentine powders. The ampoules were then plugged with gauze and the dentine-glucose pastes freeze-dried overnight. The solid masses obtained were broken down to coarse powders and distributed along the walls of the ampoules. These were then placed in large stoppered boiling tubes containing a sulphuric acid/water mixture giving a relative humidity value of 70 per cent. The tubes were then placed in an incubator at 37°C for various periods of time. The products were collected, repeatedly washed with water to remove excess glucose, and finally washed with acetone and air dried.

Treatment of sound dentine with glucosamine. 50 mg samples of demineralized sound dentine were treated with glucosamine at a relative humidity of 70 per cent, using the same procedure as described for treatment with glucose. A 30% solution of D(+) glucosamine, pH 8.5, was used.

Treatment of sound dentine with fermentation intermediates and carbohydrate degradation products. Demineralized sound dentine particles were treated with solutions of D-glyceraldehyde, dihydroxyacetone, methylfurfuraldehyde and 5-hydroxy methylfurfuraldehyde in a manner similar to that described by DREIZEN *et al.* (1958). 50 mg samples of sound dentine powder were weighed out into test tubes and 5 ml of 1% solutions of the appropriate carbohydrate derivative added. A crystal of thymol was added to each tube, and these were then plugged with cotton wool and incubated for 14 days at 37°C. The products were collected, thoroughly washed with water, acetone treated and air dried.

Measurement of collagenase susceptibility. The rate of breakdown by collagenase of each of the sound dentine preparations was determined using the method previously described (ARMSTRONG, 1958) with the exception that Jacob's modified ninhydrin method (JACOBS, 1956) was used in preference to the method of YEMM and COCKING (1955) previously employed. In the experiments where the undecalcified particles were treated with glucose solutions the particles were demineralized with EDTA prior to investigations on their susceptibility to collagenase attack.

Determination of the basic amino acid components in acid hydrolysates of the various sound dentine preparations. Quantitative analysis of the individual basic amino acids present in the various dentine hydrolysates was carried out using conventional Moore and Stein ion-exchange column chromatography with 15 cm columns (ARMSTRONG, 1961). The modified ninhydrin procedure of JACOBS (1956) was employed for the assay of the amino acid fractions eluted from the columns. Hydrolysates of each dentine product were prepared as described previously (ARMSTRONG, 1961). In addition a preparation of demineralized sound dentine was hydrolysed in the presence of 50 per cent of its own weight of sucrose to determine whether the presence of carbohydrate at this concentration had any destructive effect on the amino acids during hydrolysis.

Determination of proline and hydroxyproline. The methods previously referred to were used (ARMSTRONG, 1961).

Carbohydrate estimations. The anthrone method (ARMSTRONG, 1960) was used for the estimation of carbohydrate in the samples examined.

RESULTS

Coloration of the sound dentine matrix

The reaction of demineralized sound dentine particles with glucose (GL) at a relative humidity of 70 per cent was accompanied by a noticeable browning of the matrix. The coloration increased in intensity with the length of the incubation period and was of a biscuit brown hue after about 3 weeks.

Prolongation of the incubation for periods of up to 3 or 4 months did not produce any further deepening of this coloration.

The mineralized sound dentine particles also reacted with glucose to form a coloured derivative, but this did not deepen in intensity beyond a pale orange hue.

The demineralized sound dentine particles were rapidly and intensely coloured when incubated with glucosamine (GN) at 37°C and a relative humidity of 70 per cent. Within 3–4 days the intensity of coloration was greater than that observed in any of the other preparations.

Treatment of demineralized sound dentine with 1% solutions of the carbohydrate degradation products, methylfurfuraldehyde (MF) and 5-hydroxymethylfurfuraldehyde (HMF) also caused browning of the matrix. This effect was more pronounced with HMF-treated sound dentine, where the matrix was very similar in appearance to carious dentine preparations.

Treatment with a 1% dihydroxyacetone (DHA) solution produced a slightly coloured matrix, while 1% glyceraldehyde (GA) gave a more marked browning of the matrix, approaching in intensity the colour of the HMF-treated dentine particles. In all these preparations the colour was not removed by extensive washing of the particles with water and acetone.

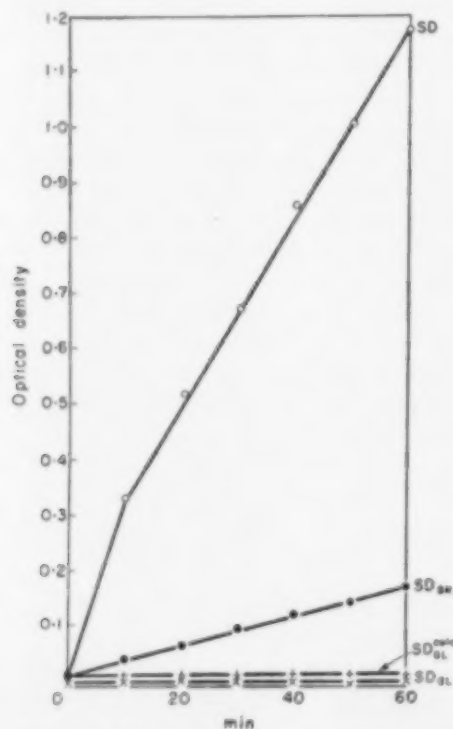


FIG. 1. Rates of attack of collagenase on the products formed between sound dentine and glucose and glucosamine. SD_{GL}^{calc} , the product formed between calcified dentine and glucose, was demineralized prior to testing for collagenase susceptibility. For other legends, see Table 1.

Susceptibility to collagenase attack

In Figs. 1 and 2 are shown the changes in susceptibility to collagenase attack induced in the dentine matrix by the various treatments described above. It was observed (Fig. 1) that the reaction of sound dentine particles—mineralized and demineralized—with glucose at 37°C, and at a relative humidity of 70 per cent, converted the matrix to forms showing complete resistance to collagenase attack.

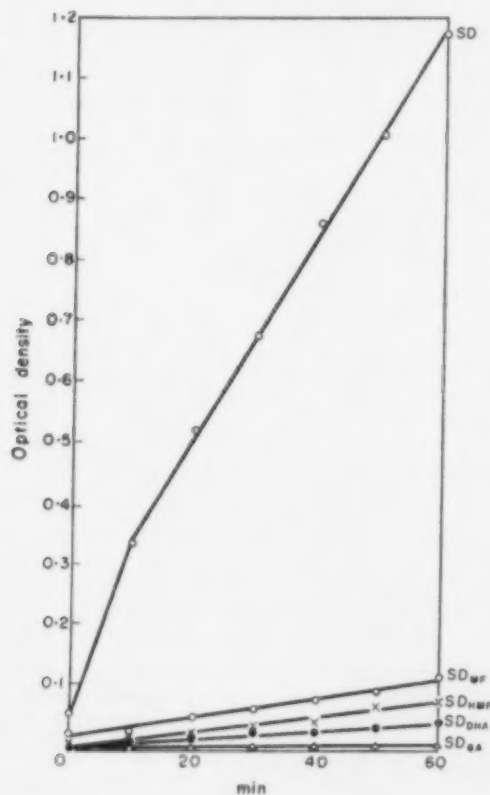


FIG. 2. Rates of attack of collagenase on the products formed between sound dentine and fermentation intermediates and sound dentine and carbohydrate degradation products. For legends, see Table 1.

Reaction with the fermentation intermediates (GA and DHA) and the carbohydrate degradation products (MF and HMF) also converted the sound dentine matrix to forms with a high degree of collagenase resistance (Fig. 2). The products showed the following order of increasing resistance to collagenase:

$$SD_{MF} < SD_{HMF} < SD_{DHA} < SD_{GA}$$

Amino acid analyses of hydrolysates of products

The results of the analyses for the imino and basic amino acids in each preparation are illustrated in the histograms in Fig. 3. These analyses show that the hydroxylysine content is reduced significantly in hydrolysates of the SD_{GA} and SD_{GL} products.

Histidine is present in reduced concentrations in the hydrolysate of the dentine-glucose product, SD_{GL} . There is more than 50 per cent loss of lysine in the hydrolysates of the SD_{GA} and SD_{GL} products, and a lesser reduction (about 20 per cent) in the SD_{HMF} product. Arginine is reduced in the SD_{GA} , SD_{DHA} and SD_{HMF} hydrolysates, but the most marked loss is in the SD_{GL} hydrolysate.

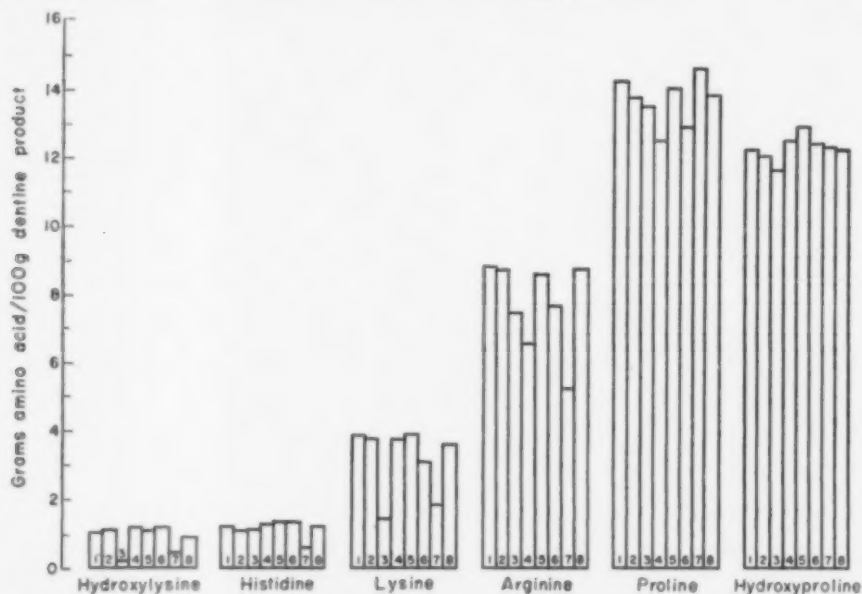


FIG. 3. Concentrations of basic and imino amino acids present in acid hydrolysates of dentine products. Results expressed as grams amino acid per 100 g dry dentine product. 1, Sound dentine (SD); 2, SD+50% sucrose; 3, SD/glyceraldehyde; 4, SD/dihydroxyacetone; 5, SD/methylfurfuraldehyde; 6, SD/hydroxymethylfurfuraldehyde; 7, SD/glucose; 8, SD/glucosamine.

There is apparently a small loss of the imino amino acids, proline and hydroxyproline, in some of the hydrolysates examined. The most significant proline loss occurred in the SD_{DHA} and SD_{HMF} products. Hydroxyproline concentration is possibly slightly less in the SD_{GA} hydrolysate. The presence of sucrose in an amount 50 per cent by weight of the matrix during hydrolysis had no destructive effect on the hydrolysate concentrations of the imino and basic amino acids.

Carbohydrate estimations

Table 1 includes the percentage "carbohydrate" content in glucose units for each preparation.

DISCUSSION

The investigation reported above was undertaken to determine whether the sound dentine matrix could react with carbohydrate and related substances to form products with properties similar to those of carious dentine. The properties of carious dentine considered were:

- (1) The brown coloration;
- (2) The resistance to collagenase attack (ARMSTRONG, 1958);
- (3) The modification of the matrix during caries, which results in reduced concentrations of imino and basic amino acids in acid hydrolysates (ARMSTRONG, 1961);
- (4) The presence of substances giving carbohydrate reactions (ARMSTRONG, 1960).

Reaction with the fermentation intermediates, glyceraldehyde and dihydroxyacetone

It was found that the demineralized sound dentine matrix reacted with aqueous solutions of two fermentation intermediates—glyceraldehyde (GA) and dihydroxyacetone (DHA)—to give dentine products (SD_{GA} and SD_{DHA}) which were strongly resistant to collagenase disintegration (Fig. 2). The SD_{GA} product was brown and not dissimilar from carious dentine in appearance, whereas the SD_{DHA} product was only faintly coloured. DHA does not give a carbohydrate reaction with anthrone: however, analysis showed 0.8 per cent "glucose" in the SD_{DHA} product, and it may be that the reaction product formed between the SD and DHA gave coloured products in the subsequent carbohydrate analysis with absorption properties at the wavelength used in the anthrone procedure for glucose estimation. The SD_{GA} product contained only a small excess of "carbohydrate" over the SD value. Acid hydrolysates of carious dentine have been shown to give lowered concentrations of arginine, proline and hydroxyproline and hydroxylysine: it is possible that lysine may be reduced also, but with the effect masked by interfering material during analysis (ARMSTRONG, 1961). Hydrolysates of SD_{GA} contain reduced concentrations of hydroxylysine, lysine and arginine (Fig. 3), and probably a small reduction in hydroxyproline content as well.

Reaction with carbohydrate degradation products—methylfurfuraldehyde and hydroxymethylfurfuraldehyde

The reaction products formed between the dentine matrix and the carbohydrate degradation substances, methylfurfuraldehyde (MF) and hydroxymethylfurfuraldehyde (HMF) were also examined for properties commonly associated with carious dentine. HMF is formed in the cooking and processing of carbohydrate-containing food and may, therefore, be present in significant amounts in civilized diets. Both the dentine products formed, SD_{MF} and SD_{HMF} , were distinctly brown and similar in appearance to carious dentine preparations. Further, both SD_{MF} and SD_{HMF} were markedly resistant to collagenase (Fig. 2).

HMF is formed when concentrated acids act on certain carbohydrates—an essential stage in many colorimetric procedures in carbohydrate analysis. It might be expected, therefore, that its reaction with the dentine matrix would increase the

"carbohydrate" content of the matrix, and on subsequent analysis the SD_{HMF} product was found to contain 1.7 per cent "glucose". MF is structurally very similar to HMF, differing only in the absence of an hydroxy group from the radical attached to the 5-carbon atom of the furan ring common to both substances. The dentine product SD_{MF} showed a "glucose" content of 0.9 per cent. Analysis of the acid hydrolysate of SD_{MF} showed the same concentrations of basic amino acids as are present in equivalent SD hydrolysates. However, the SD_{HMF} hydrolysate showed lowered concentrations of lysine, arginine and, to a lesser extent, proline.

Reaction with glucose and glucosamine

The reaction between carbohydrates and proteins has been investigated in considerable detail by LEA and his co-workers (1949-1952). They showed that reaction between casein and glucose involved the lysine ϵ -amino groups in the protein, and that arginine and histidine units were also involved; acid hydrolysates of the casein/glucose products contained reduced amounts of lysine, arginine and histidine (LEA and HANNAN, 1950b). The product of this casein-glucose reaction was brown. In the product formed between casein and glucosamine the brown colour developed more rapidly and intensely than with the casein-glucose product, and analysis showed no involvement or destruction of the lysine side chains.

Investigations on the products formed between the collagenous dentine matrix and glucose and glucosamine respectively showed many similarities to those reported by LEA and HANNAN for their casein products. Both the SD_{GL} and SD_{GN} products were brown, but the coloration was more rapid and intense in the SD_{GN} product. Amino acid analyses showed fairly extensive destruction of hydroxylysine, histidine, lysine and arginine in the SD_{GL} product, but no such losses were observed in the SD_{GN} hydrolysate. These features are similar to those found in the casein-glucose and casein-glucosamine products. In addition, the SD_{GL} product was completely resistant to collagenase, and the SD_{GN} also showed a considerable degree of resistance to the enzyme attack. The SD_{GL} product contained 1.6 per cent "glucose" and the SD_{GN} product contained 0.6 per cent "glucose". Since the anthrone method gives no colour reaction with glucosamine itself, and LEA and RHODES (1952) found no recoverable glucosamine in hydrolysates of their casein-glucosamine products, the 0.6 per cent "glucose" value found for the SD_{GN} product cannot necessarily be interpreted as indicating the absence of significant concentrations of glucosamine-derived carbohydrate material in the preparation. The properties of the various dentine products discussed above are summarized in Table 1 and compared with the properties of carious dentine.

On the basis of these observations it may be concluded that reactions of the dentine matrix with the various carbohydrate substances and derivatives examined can modify the matrix to forms with many of the properties associated with carious dentine. All the substances induced a degree of collagenase resistance in the matrix as a result of their reaction with sound dentine. Similarly each dentine product was coloured brown as the result of its reaction with the carbohydrate derivative concerned, and many of the products were similar to carious dentine in appearance.

TABLE 1. SUMMARY OF PROPERTIES OF DENTINE PRODUCTS

Dentine product	Coloration of matrix*	Resistance to collagenase†	Amino acid reduction in acid hydrolysates‡						Carbohydrate content (% "glucose")
			HyLys.	Lys.	His.	Arg.	Pro.	Hypro.	
CD	+++	1	+	+?	0	++	++	++	4.0
SD _{GA}	++	2	+++	+++	0	+	0	0?	0.5
SD _{DHA}	+	2	0	0	0	+	+	0	0.8
SD _{MF}	+++	2	0	0	0	0	0	0	0.9
SD _{HMF}	+++	2	0	+	0	+	+	0	1.7
SD _{GL}	+++	3	+++	+++	+++	+++	0	0	1.6
SD _{GN}	++++	1	0	0	0	0	0	0	0.6
SD	0	0	0	0	0	0	0	0	0.4

* Coloration of matrix powder: 0, colourless; +, pale orange; ++, light brown; +++, brown (\approx CD); +++++, dark brown.

† Resistance to collagenase: 0, maximal breakdown (= SD); 1, partially resistant; 2, highly resistant; 3, completely resistant.

‡ Amino acid loss in hydrolysates: 0, no loss (=SD); +, 5-14 per cent loss; ++, 15-34 per cent loss; +++, greater than 35 per cent loss.

Abbreviations: SD (Sound dentine), CD (Cariou dentine), GA (Glyceraldehyde), DHA (Dihydroxyacetone), MF (Methylfurfuraldehyde), HMF (Hydroxymethylfurfuraldehyde), GL (Glucose), GN (Glucosamine), HyLys. (Hydroxylysine), Lys. (Lysine), His. (Histidine), Arg. (Arginine), Pro. (Proline), Hypro. (Hydroxyproline).

The exceptions were the SD_{GA} product, and the matrix product formed in the reaction of glucose with the calcified sound dentine, both products being only pale orange in appearance.

The reactions between sound dentine and glucose and sound dentine and glucosamine were carried out at a relative humidity of 70 per cent in contrast to the aqueous conditions employed for the other preparations. LEA and HANNAN (1949) showed that the casein-glucose reaction was critically sensitive to the moisture content of the environment, and that under aqueous conditions no "browning reaction" took place. In preliminary investigations (ARMSTRONG, unpublished) it was found that prolonged incubation of sound dentine particles with 30% aqueous solution of glucose did not induce any matrix coloration. However, the results reported above show that sound dentine reacts under aqueous conditions with HMF, MF and GA to form products similar in colour and intensity to CD and the SD_{GL} product. Although it is difficult to assess the environmental conditions which may be operating at the molecular level in the dentine during caries, it is possible that direct reaction between the matrix and sugars, and sugar derivatives, may occur and give rise to a browning coloration even if conditions were not optimal for these reactions.

The sound dentine-glucose product (SD_{GL}) showed the most marked reduction in basic amino acid content (hydroxylysine, histidine, lysine and arginine), and also the greatest collagenase resistance. There was little evidence of any extensive loss of the imino amino acids, proline and hydroxyproline, in acid hydrolysates of the products examined. Small losses in proline may have occurred in SD_{DHA} and

SD_{HMF} hydrolysates, but if so, to a much less extent than the losses found in carious dentine hydrolysates. When sound dentine is hydrolysed in the presence of 50 per cent of its own weight of sucrose, the resultant hydrolysate contains the basic and imino amino acids in equivalent concentrations to those found for sound dentine alone. Sucrose was chosen because it would be initially rapidly hydrolysed to glucose and fructose, and so provide a means of testing whether the presence of either aldohexose or ketohexose sugars during hydrolysis would lower the basic amino and imino acid content in the hydrolysate. Since no such effect was observed in the presence of 50 per cent sucrose it seems unlikely that the mere presence of 1.6 per cent "glucose" (in SD_{GL}) or 4 per cent "glucose" (in CD) can account for the losses in amino acid content observed in these and similar dentine product hydrolysates, but rather that such losses are the result of the actual reactions involved in the formation of the dentine-carbohydrate compounds. In view of these results and the presence of 4 per cent carbohydrate in carious dentine samples (ARMSTRONG, 1960) it is possible that reaction of the dentine matrix with carbohydrate substance during the carious process may be the cause of the browning of the matrix, the collagenase resistance, and the arginine (and possibly lysine?) modifications associated with carious dentine. However, such a carbohydrate-dentine reaction could not apparently account for the losses of proline and hydroxyproline observed in acid hydrolysates of carious dentine. In this latter context it is of interest to note that MEILMAN, GALLOP and SEIFTER (1958) in their studies on the action of bacterial collagenase on the soluble collagen, ichthyocol, observed the release of peptides rich in proline and hydroxyproline. If, as previously discussed (ARMSTRONG, 1961), some degree of enzymic proteolytic degradation of the dentine matrix occurs during caries, it is highly probable that this is facilitated through the action of bacterial collagenases. It can be postulated that such action, which could account for the lowered proline and hydroxyproline concentrations found in carious dentine, occurs simultaneously with, or in close antecedence to, the reaction of the dentine with carbohydrates or derivatives of the types considered above, these latter reactions (i) rendering the residual matrix resistant to further collagenase attack, (ii) turning it brown, and (iii) affecting the basic amino acid components present. This combination of events could provide an explanation of the observed properties of carious dentine.

Acknowledgement—It is a pleasure to record the high standard of technical assistance given by Miss BRITA ERHOLTZ in this investigation.

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THE EFFECT OF HYPER-VITAMINOSIS A ON THE DEVELOPMENT OF RAT TOOTH GERMS IN TISSUE CULTURE

P. J. HOLLOWAY* and MAY MELLANBY
Medical Research Council, London, England

Abstract—Pairs of first molar tooth germs from 17-day rat embryos were cultured *in vitro*, one member of the pair having additional vitamin A added to the medium on which it was grown. The germs cultured on hyper-A medium were smaller than the control teeth, and lagged in cusp development. The histodifferentiation of the enamel organs was severely affected, but the dental papillae showed less differences. The changes were almost completely reversible up to 7 days of culture.

INTRODUCTION

THE DEVELOPMENT of explanted tooth germs *in vitro* was first described by GLASSTONE (1935). Since then the technique of organ culture has been used mainly to elucidate the mechanism of normal tooth development, while the factors affecting this have, for the most part, been ignored. The techniques of culture have followed closely those developed by FELL and ROBISON (1929) for the growth of bone rudiments, and subsequently used to great effect by FELL and MELLANBY (1952) in the study of factors affecting this growth. It was felt that the method lent itself to an investigation of factors which might influence the development of tooth germs. The first of these to be tested was the effect of adding additional vitamin A to the culture medium.

EXPERIMENTAL METHOD

Mandibular first molar tooth germs were dissected from 17-18 day rat embryos. At this stage the germs were bell-shaped (Fig. 1), and consisted of an internal and an external enamel epithelium separated by a primitive type of stellate reticulum (Fig. 2). The inner enamel epithelium was several layers thick and consisted of spherical cells. Each tooth-germ contained a dental papilla which showed no cellular differentiation.

The dissected germs were placed on medium made from cock plasma clotted by the addition of the tissue extract of a 13-day incubated chick embryo, and transplanted to a fresh clot three times a week. A tooth germ from one side of the jaw was placed on control medium while that from the other side was placed on medium made with plasma to which additional vitamin A had been added. The vitamin A used was in the alcoholic form, and was suitably diluted with ethanol and added to the plasma to produce approximately 1350 i.u. per 100 ml. An equivalent amount

* Present address: London Hospital Medical College, Whitechapel, London E.1.

of ethanol was added to the control plasma thus making the vitamin A content the only variable. The method of preparation has been fully described by FELL and MELLANBY (1952).

At the time of explant, two tooth germs from each litter of embryos were dissected and immediately fixed, sectioned and stained to ascertain the stage of development attained by that particular litter. Pairs of control and experimental germs were fixed at 2, 3, 4, 7 and 14 days of culture, to compare their development at these various stages. The tooth germs were fixed in 3% acetic Zenker's fluid, sectioned at 7 μ and stained with Delafield's haematoxylin and Biebrich's scarlet and eosin.

RESULTS

Periodically the tooth germs were examined under a dissecting microscope and measured in arbitrary units in two directions at right angles. After only 4 days of culture it was noticed that the experimental germs were not growing as quickly as the controls, and by 7 days there were obvious differences in size (Table 1). After 14 days, when the experiments were terminated, the two sets of germs bore no resemblance to each other. Cuspal development was obvious in the control germs, but was not discernible in the germs developing on the hyper-A medium.

TABLE 1. PERCENTAGE INCREASE IN LENGTH OF TOOTH GERMS DURING 14 DAYS CULTURE

Days of culture	No. of germs	Increase in size (%)	
		Control	Hyper-A
2	31	12.5	10.0
4	29	30.0	12.5
7	19	35.0	20.0
11	11	56.0	10.0
14	8	75.0	0.0

Differences were apparent between the two groups when examined histologically. The developmental changes that occur within these cultures when placed on normal media have been well described by GLASSTONE (1935), and the control germs of the present investigation followed this pattern closely. For the first few days the explants developed rapidly, and soon presented the appearance of fully differentiated tooth germs. The inner enamel epithelium developed into a single layer of columnar-shaped ameloblasts, and their nuclei migrated to the distal poles of the cells. At the same time odontoblasts differentiated on the surface of the dental papilla, and laid down some typically tubular dentine matrix (Fig. 3). During this time a very well developed cuspal pattern appeared (Fig. 4). 14 days of culture *in vitro* had produced the equivalent of about 5 days development *in vivo*.

After 4 days of culture, the control and experimental germs had differentiated to approximately the same extent. Cusps had developed in both, and the ameloblasts

were forming, although the internal enamel epithelium was still several cells thick. The stratum intermedium and stellate reticulum also appeared more mature.

The first stage at which differences in differentiation were noticed occurred at about 7 days of culture (Fig. 5). Further cuspal development could still be seen in both groups, but was not so marked in the hyper-A germs. The ameloblast layers in the controls were at a more advanced stage of development; their cells were more columnar and the nuclei had migrated further towards the distal poles of the cells.

Fig. 6 shows the condition after 14 days when there was a marked difference. The control germs have already been described, and it will be seen that the hyper-A germs had a completely different appearance. Cusp formation had regressed, and a higher power view (Fig. 7) shows that the ameloblasts were spherical or short columnar cells with their nuclei centrally placed. Very little of the rest of the enamel organs remained. The dental papillae were large and, although some odontoblasts had formed and a very small amount of early dentine matrix had been laid down, the development had obviously lagged a considerable way behind the control germs. No degenerative changes were seen in the cells of the dental papillae of either the control or the experimental germs.

In order to test whether these changes were irreversible, a second experiment was carried out, similar to that already described except that some of the tooth germs, after being kept on the hyper-A media for 7 days, were transferred to control media for a further 7 days. Tooth germs were fixed at explant, and at 7 and 14 days of culture. After 7 days differences between the control and experimental germs similar to those already described were reproduced. Those maintained on hyper-A media for a further 7 days confirmed the changes described. However, those germs that were transferred from the hyper-A to the control media showed considerable differences. Not only cusp formation but also cell morphology appeared to be more normal; the ameloblasts looked more typical and the odontoblasts had laid down a fair amount of dentine matrix. Growth was not affected to the same degree, the average size of the transferred tooth germs being a little less than the controls.

DISCUSSION

Vitamin A is one of several nutritional factors that have been shown to affect the development of teeth (WOLBACH and HOWE, 1933; SCHOUR, HOFFMAN and SMITH, 1941; MELLANBY, 1941; SALLEY and BRYSON, 1957). From our knowledge of the effects of vitamin A upon epithelial tissue and bone, an effect upon teeth is perhaps to be expected. However, reported investigations on teeth have involved studies *in vivo*, and none have attempted to define the nature of the influence. FELL and MELLANBY (1952, 1953) clearly demonstrated that the action of the vitamin on bone and epithelium was, at least in part, a direct one. It would seem from the present study that the same is true of its action upon developing teeth.

The effect of hypervitaminosis A was first noticeable on the growth of the tissue. A lag in growth was detected as early as the fourth day of culture, whereas histodifferentiation was not apparently affected until after this time. Another

interesting observation was the regression of cusp formation that occurred under the influence of the additional vitamin A. After 4 days of culture the experimental teeth were at the same stage of cusp formation as the control germs, but at 7 days the latter had developed much more obvious cusps, whereas the hyper-A germs seemed to have advanced little further than after only 4 days of culture. After 14 days the control teeth had a fully mature cusp morphology, while the experimental germs had lost all semblance of cusps, and were more or less rounded in outline.

Histologically, the enamel organ was much more radically affected than the dental papilla. It has been suggested that the size and shape of a tooth is under the control of the ectodermal tissue, which also influences the differentiation of the odontoblasts. In this study, the ameloblasts of the hyper-A germs developed into a single row of cuboidal cells, while the rest of the enamel organ lost its character altogether. However, apart from some slight changes in the odontoblasts, and a lack of formation of a substantial amount of dentine matrix, there were no obvious differences between the dental papillae of either group. It would appear that this is another example of vitamin A directly modifying an epithelial tissue, the changes in the mesodermal tissue being possibly secondary in this particular instance. It is interesting to note that these changes seem to be almost completely reversible, at least up to 7 days of culture.

It is important to emphasize that rat plasma normally has a vitamin A concentration of about 60–100 i.u. per 100 ml, but cock plasma has about 200–300 i.u. per 100 ml, so that even the control germs in this study were growing on hyper-A media. Cock plasma was used because it is convenient from many aspects, but an attempt was made to grow tooth germs on plasma obtained from dogs whose plasma concentration was approximately 80 i.u. per 100 ml. However, this proved a failure as the clots were more liable to undergo liquefaction and the tooth germs died. With improved technique, these difficulties might well be overcome.

Even under the most favourable conditions, the ameloblasts stopped their development at the stage of full histodifferentiation and never produced enamel matrix. The reason for this is not understood, but may be connected with the unidirectional nutritional supply that exists following the first deposition of dentine matrix. Several methods were tried in an effort to improve culture conditions but, despite the fact that growth rate was considerably improved by placing the germs on sterile net rayon before putting them on clots (SHAFFER, 1956), no later stage of development was reached than that already described. It is of interest that HAY (1961) describes the production of enamel matrix in mouse tooth germs cultivated in a similar manner to the rat material used in this study.

No doubt other nutritional factors may affect the development of tooth germs in this experimental medium. The effect of excess of vitamin D has already been tested. This vitamin had no apparent effect after 14 days of culture under the conditions of the test. Vitamin D₂ was added to the medium to a final concentration of 700 i.u. per 100 ml. However, this result does not exclude the possibility that excess of the vitamin may have an indirect effect *in vivo*, or that it may affect development at a later stage, especially during calcification.

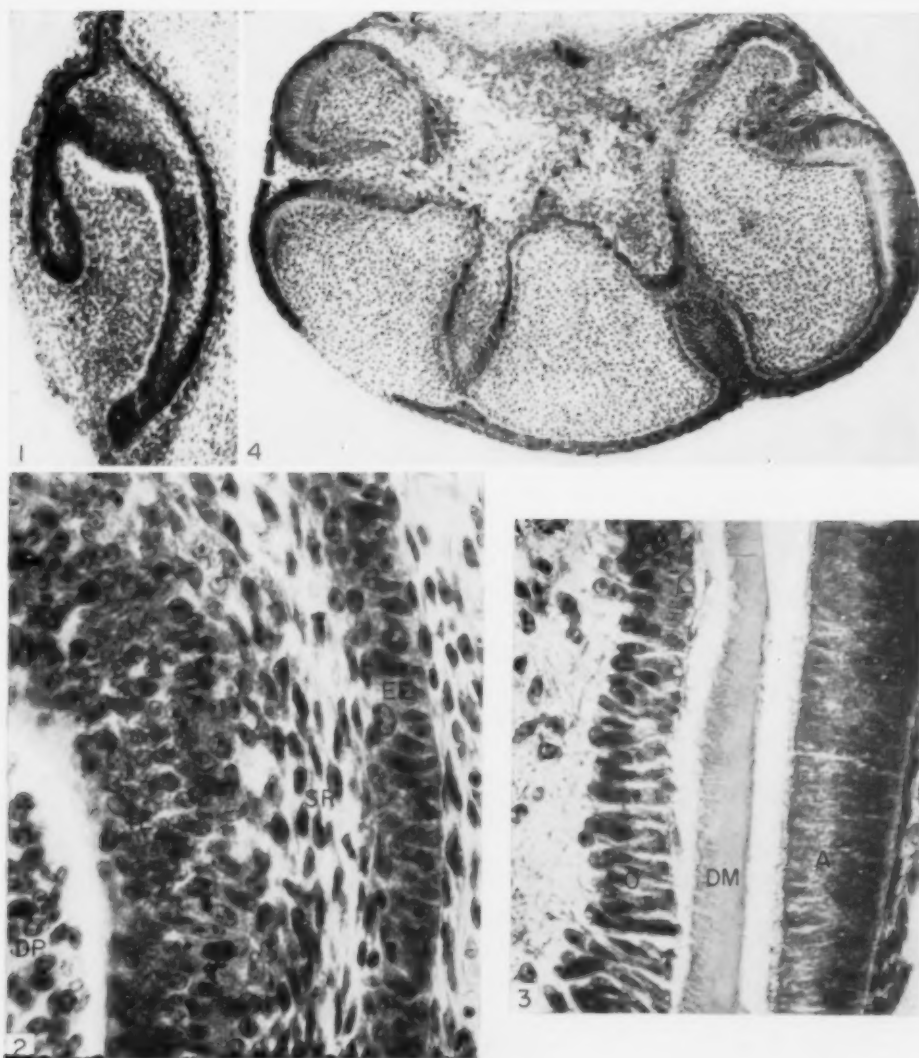
This particular method lends itself to a study of the effect of many other factors both within and outside the nutritional field. At the moment it is limited to the effect of excess rather than deficiency, but the latter may also come within its scope with the further development of synthetic culture media.

Acknowledgements—The authors wish to thank the Medical Research Council of Great Britain for financing this research project, and also Mr. R. J. C. STEWART for his help in the technical procedures.

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EFFECT OF HYPER-VITAMINOSIS A ON DEVELOPMENT OF RAT TOOTH GERMS



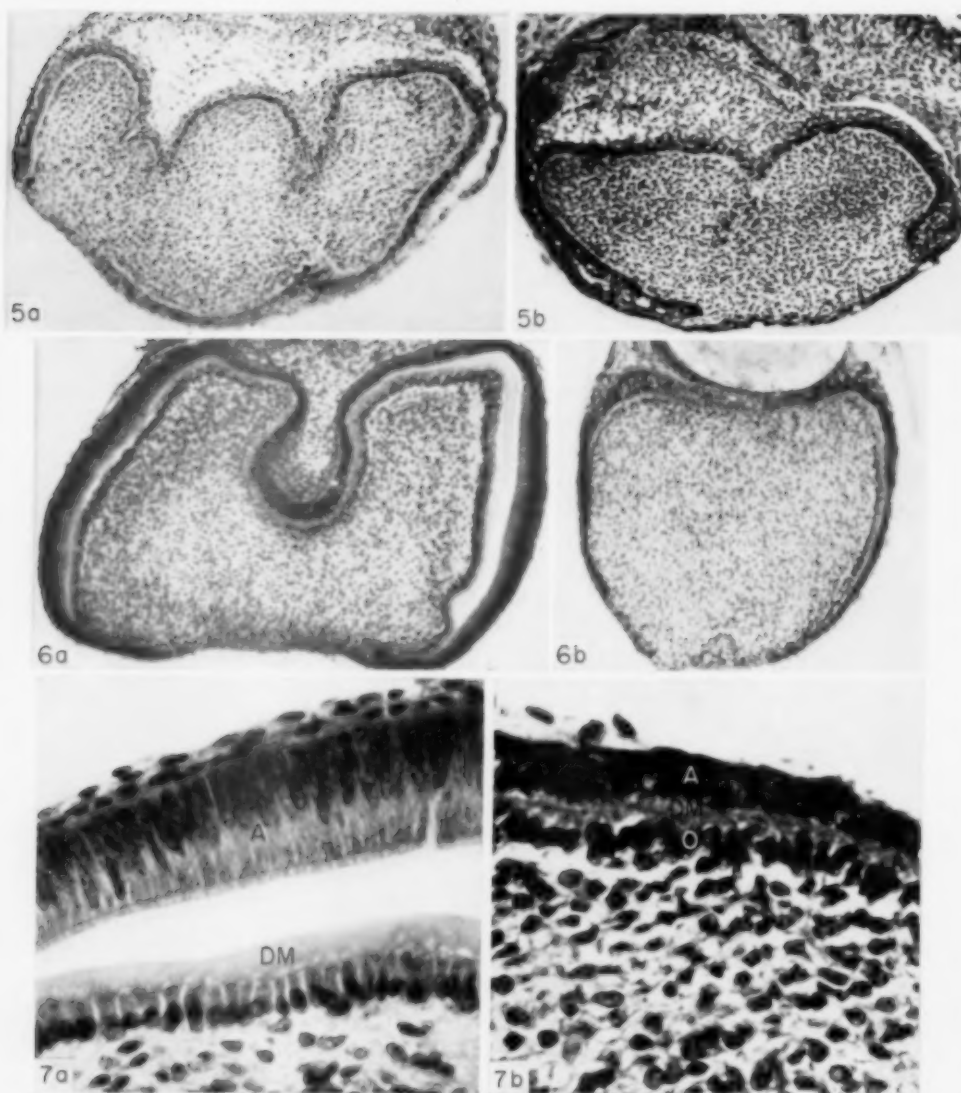
All the sections were stained with haematoxylin and eosin

FIG. 1. Tooth germ at explant. $\times 80$.

FIG. 2. Tooth germ at explant. EE, external enamel epithelium; IE, internal enamel epithelium; SR, stellate reticulum; DP, dental papilla. $\times 500$.

FIG. 3. Tooth germ after 14 days of culture on normal medium. A, ameloblasts; DM, dentine matrix; O, odontoblasts. $\times 500$.

FIG. 4. Cross-section through cusps of tooth germ after 14 days of culture on normal medium. $\times 80$.



All the sections were stained with haematoxylin and eosin.

FIG. 5. Tooth germ after 7 days of culture on (a) normal and (b) hyper-A medium. $\times 80$.

FIG. 6. Tooth germ after 14 days of culture on (a) normal and (b) hyper-A medium. $\times 80$.

FIG. 7. Tooth germ after 14 days of culture on (a) normal and (b) hyper-A medium. A, ameloblasts; DM, dentine matrix; O, odontoblasts. $\times 500$.

Vol.
5
1961

FURTHER OBSERVATIONS ON THE EFFECT OF SURGERY ON THE MENISCUS OF THE MANDIBULAR JOINT IN RABBITS

R. SPRINZ

Department of Oral Anatomy, University of Sheffield, England

Abstract—Disks were studied histologically, 8 weeks after unilateral operations. Damage to the meniscus resulted in repair only if the damage was minimal and related to the periphery. When after surgical operation the meniscus remained divided, condylar overgrowth occurred similar to that previously described for complete meniscectomies. Control operations in which the joint cavities were merely opened led to little change of the articular surfaces, except for a small reduction in the cartilage covering of the condyle.

INTRODUCTION

IN 1937, DUBECQ gave a detailed account of complete meniscectomy in rabbits. He also traumatized the meniscus. His observations indicated that following complete meniscectomy no regeneration of the disk occurred within 6 months and that after traumatizing the disk it sometimes disintegrated completely.

In a previous paper (SPRINZ, 1954) DUBECQ's findings were confirmed, but it was found that the articular surfaces of the joint became enlarged, a condition which appeared to be progressive even at 32 weeks after operations. The operations were performed bilaterally and unilaterally, and assessment of masticating efficiency was noted.

The present paper is an account of a study of the regenerative capacity of the disk.

METHODS AND MATERIALS

Rabbits approximately 6 weeks old were used. They were laboratory animals of no particular strain.

The animals were anaesthetized with Nembutal (40 mg per 1 kg body wt.) injected into the lateral vein of the ear.

The surgical approach to the joint was similar to the one described for the complete meniscectomy (SPRINZ, 1954).

The incision was made from the lateral canthus of the eye to the external auditory meatus—the landmark being the backward projection of the zygomatic arch, 0.5 cm above which the condyle of the mandible can be felt. The line of incision was curved, with the concavity directed inferiorly. This approach prevented damage to the facial nerve, though the superficial temporal vein was not infrequently cut.

1. *Partial meniscectomy* (Fig. 1)

Once the disk was clearly visible at the posterior aspect of the mandibular joint, resting on the condyle, it was subjected to either:

(a) an antero-posterior incision on its superior surface, though not through the whole thickness of the disk, extending approximately from the centre to the periphery of the disk at its posterior aspect (PM 7 and 8); this operation involved the superior joint cavity only.

(b) a division of the disk, similar to (a) but made through the whole thickness of the disk (PM 5 and 6), so both joint cavities were involved.

(c) a removal of approximately one quarter of the disk from the posterior aspect (PM 9 and 10), or

(d) an excision of approximately one half of the disk from the posterior aspect (PM 11 and 12).

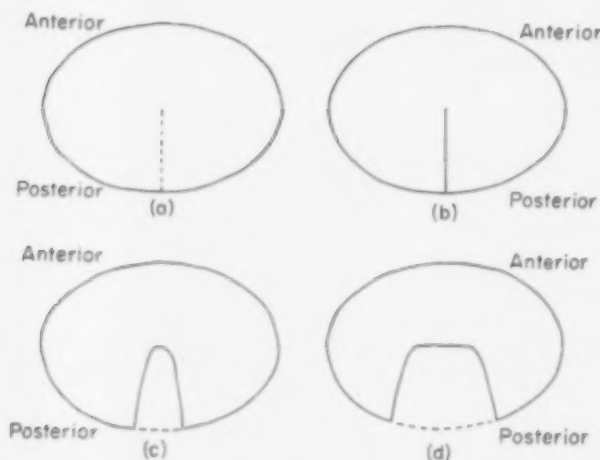


FIG. 1. Partial meniscectomy; types of operation. (a) Incision into the disk on its superior surface (though not through it), running approximately from the centre to the periphery of the disk. The direction of the cut is antero-posteriorly. (b) Division of the disk along a line similar to (a), the cut being made through the whole thickness. (c) Excision of approximately one quarter of the disk from the posterior aspect. (d) Excision of approximately half of the disk from the posterior aspect.

Eight animals were used (two for each type of operation); the operations were performed unilaterally, and the experimental period was 8 weeks.

The animals were killed by an overdose of Nembutal. After disarticulating the mandibles the disks were dissected from the condyles, fixed in formol saline, serially sectioned and stained with haematoxylin and eosin. The sections were cut in a medio-lateral plane, passing approximately at right angles to the zone of operation. The mandibular condyles were photographed immediately after removal of the disks.

2. Control operations

The joint was approached as for the partial meniscectomy series described above. When the capsule of the joint was recognized a fine-pointed scalpel was introduced without damage to the disk, either into the superior joint cavity, or into the inferior joint cavity, or into both joint cavities.

The synovial fluid was allowed to escape and the wound was sutured. The operations were performed unilaterally only. In all nine animals were used; two had control operations performed on the superior joint cavity (CO 1 and 4), three were subjected to operations on the inferior compartment (CO 2, 5 and 10), and four had both joint cavities operated at the same time (CO 3, 6, 11 and 12). The animals were sacrificed 8 weeks after operation.

The mandibles were disarticulated and after removal of the disks, the condyles were examined and photographed. One joint (CO 11) which was subjected to operations on both joint cavities was fixed in formol saline, decalcified in a mixture of formic and citric acid, cut in serial sections, and stained with haematoxylin and eosin.

RESULTS

1. Partial meniscectomy

The average weight of the eight animals before operation was 950 g, at termination of the experiment the animals weighed on average 2370 g. This represents a mean weekly increase of 177.5 g.

Microscopic appearance. (a) In both animals (PM 7 and 8) used in the incision experiment, some fibrous repair was seen. One animal (PM 7) presented a superficial niche at the periphery (Fig. 2), which led to an extensive gap at the centre of the disk (Fig. 3).

(b) In the division experiments the disk of one animal (PM 5) showed evidence of repair; near the periphery the disk became vascularized and the region of the cut was repaired by fibrous tissue (Fig. 4); nearer the centre of the disk the vascularization was less marked but evidence of fibrous repair was still visible (Fig. 5); at the centre a small gap was still patent. In the other experiment (PM 6) the main part of the operated zone showed no evidence of repair; the divided parts of the disk remained separate and one of the stumps contained a small area of bone formation (Fig. 6).

(c) In both experiments involving the excision of a quarter of the disk, no regeneration was observed and in both cases one of the stumps showed cartilage cell and bone formation. In one animal (PM 9) an extensive pyramidal piece of bone was formed in the larger stump of the disk, the bone appearing to have been formed by endochondral ossification (Fig. 7).

(d) In the two animals in which one half of the disk was removed, no regeneration was observed. Indeed in one specimen no part of the disk was recovered at autopsy. In the other specimen the extent of the gap closely corresponded to the excised area.

Articular surfaces after operations. In the animals in which incision and division of the disk were followed by active repair, the condylar surface remained close to the normal limits. In one of the division experiments and in all partial excision

experiments the articular surface of the condyle increased markedly in size and presented indentations (Fig. 8), resembling closely the condition following complete meniscectomy previously described.

2. Control operations

The average weight of the nine animals before operation was 930 g, at termination of the experiment the animals weighed on average 2440 g. This represents a mean weekly increase of 188.7 g.

Macroscopic appearance (Fig. 8). Following operations involving the inferior compartment the condylar surfaces underwent little change. In relation to the area of incision a slight erosion was noted, and in one case (CO 12) small excrescences were also seen; however the changes were minimal.

No abnormality of articular surfaces was noted in the animals on which only the superior compartment operations were performed.

Microscopic appearance. The erosions observed macroscopically were seen to be related to a reduction of the cellular cartilage layer which in some areas was completely absent (Fig. 9). The fibrous articular membrane, however, was intact and thicker than normal, but no bony spicules appeared on the articular surface. The joint was completely normal in relation to the unoperated part. The condyle of an unoperated joint is seen in Fig. 10.

DISCUSSION

The mandibular meniscus in the rabbit showed little regenerative power. Only if the damage to the disk was minimal and related to the periphery (i.e. close to the area of vascular supply) was any repair observed. This took the form of vascularization followed by fibrous repair. If the damage to the disk was more extensive or if a small piece of disk was removed, no regeneration occurred but areas of cartilage cells, which are not normally found in the meniscus of the rabbit, and areas of calcification appeared.

These results differ materially from those described by WALMSLEY and BRUCE (1937) for the menisci in the knee joint of the rabbit. They showed that 22 days after excision a new disk formed.

The appearance of calcified zones related to areas of necrosis in the disk in man has been demonstrated by BAUER (1932). He ascribed this to degenerative joint disease. This explanation does not apply to similar areas observed in the animal experiments described above. It appears likely that these areas are the direct consequence of a localized ischaemia resulting from the operative procedure as well as a traumatic effect following alterations in the pressure on the disk.

When the disk damage was not repaired, condylar overgrowth occurred, the results closely resembling the meniscectomy series (SPRINZ, 1954). DUBECQ (1937) describing the results of trauma to the disk, stated that the articular surface presented some slightly rough places on the surface ("quelques légères aspérités de surface") and some indentation. The results of the present series bear out the findings of

DUBECQ, who kept his animals for 30 weeks, though irregularities and indentations were associated with marked condylar overgrowth within 8 weeks of operation.

DUBECQ stated that in one of his animals the trauma to the disk led to its complete disintegration. This phenomenon was also observed in one animal of the present series in which one half of the disk was excised.

Removal of, or damage to, the disk—providing repair did not occur—led to condylar enlargement. The control operations in which the synovial fluid was released from the joint cavities led to a reduction in size of the mandibular condyle. It appears, therefore, that overgrowth of the condyle is related to changes within the joint (i.e. the disk) and not to the operative procedure of opening the joint surgically.

The operations led to no disharmony in masticatory function. The persistently growing teeth were worn at an even rate, and the average weekly increase in weight of the partial meniscectomy series was only 11.2 g below that of the control group, a difference which is of no significance.

Acknowledgements—The author wishes to thank Miss MARJORIE WEDGWOOD for her helpful suggestions and Mr. I. F. COOMBE and Mr. R. COUSINS for their technical assistance. He also wishes to record his appreciation of the generous assistance of the University of Sheffield which provided him with a research grant to carry out this investigation.

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PLATE 1

FIG. 2. Disk showing superficial niche on superior surface close to the periphery of the disk—8 weeks after incision experiment (a)—(PM 7). Haematoxylin and eosin. $\times 70$.

FIG. 3. Same disk as Fig. 2, showing gap on superior surface close to the centre of the disk. Haematoxylin and eosin. $\times 70$.

FIG. 4. Disk showing blood vessels in operation zone close to the periphery of the disk—8 weeks after division experiment (b)—(PM 5). Haematoxylin and eosin. $\times 70$.

FIG. 5. Same disk as Fig. 4, showing fibrous repair close to the centre of the disk. Haematoxylin and eosin. $\times 70$.

FIG. 6. Disk which has remained divided following operation, the smaller stump showing an area of calcification—8 weeks after division experiment (b)—(PM 6). Haematoxylin and eosin. $\times 18$.

Fig. 7. Stump of disk in which a triangular piece of bone (B) can be seen. Cartilage cells are seen at (C), the fibrous stroma of the disk at (F)—8 weeks after excision of one quarter of the disk experiment (c)—(PM 9). Haematoxylin and eosin. $\times 20$.

EFFECT OF SURGERY ON MENISCUS OF RABBIT MANDIBULAR JOINT

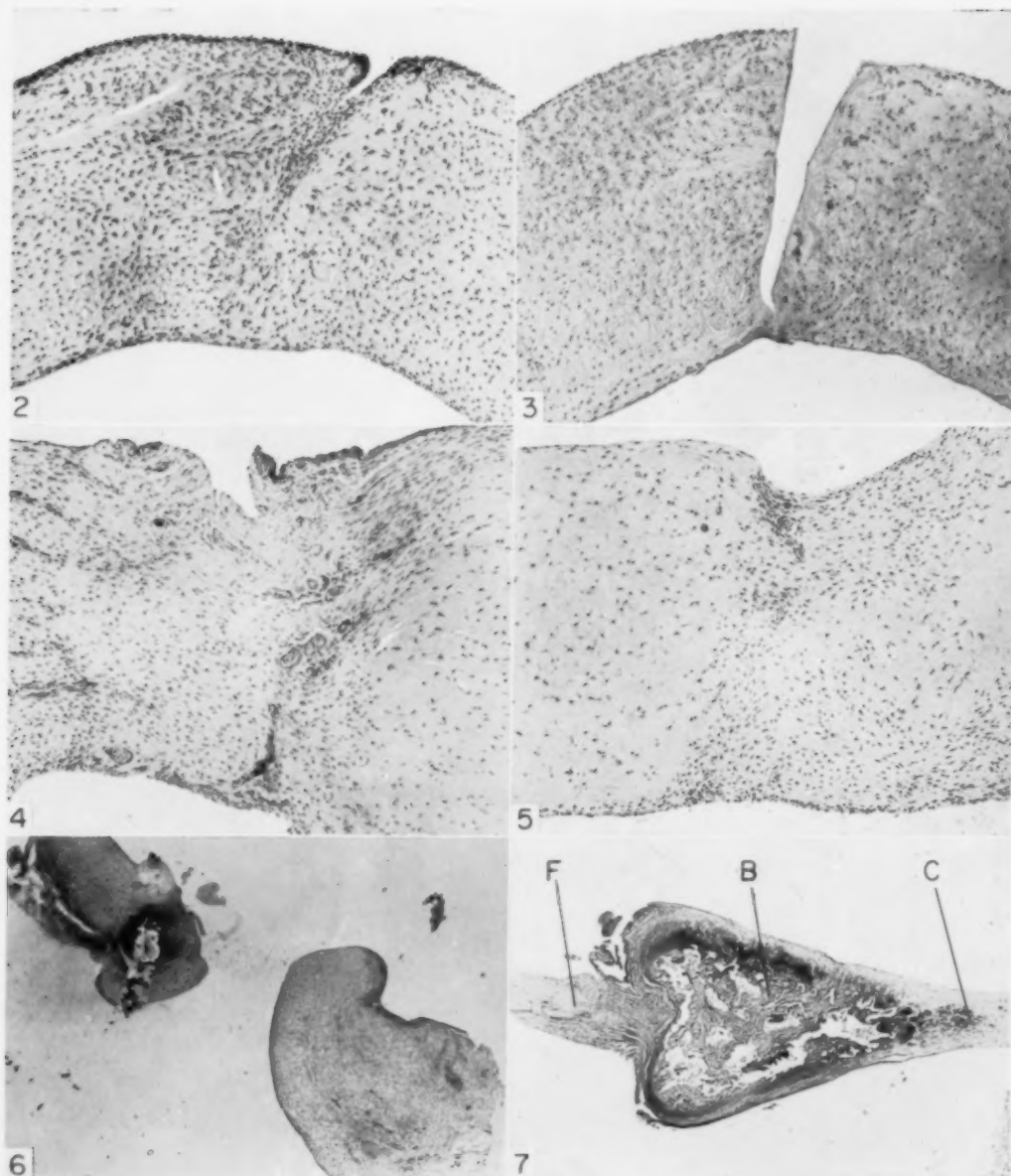
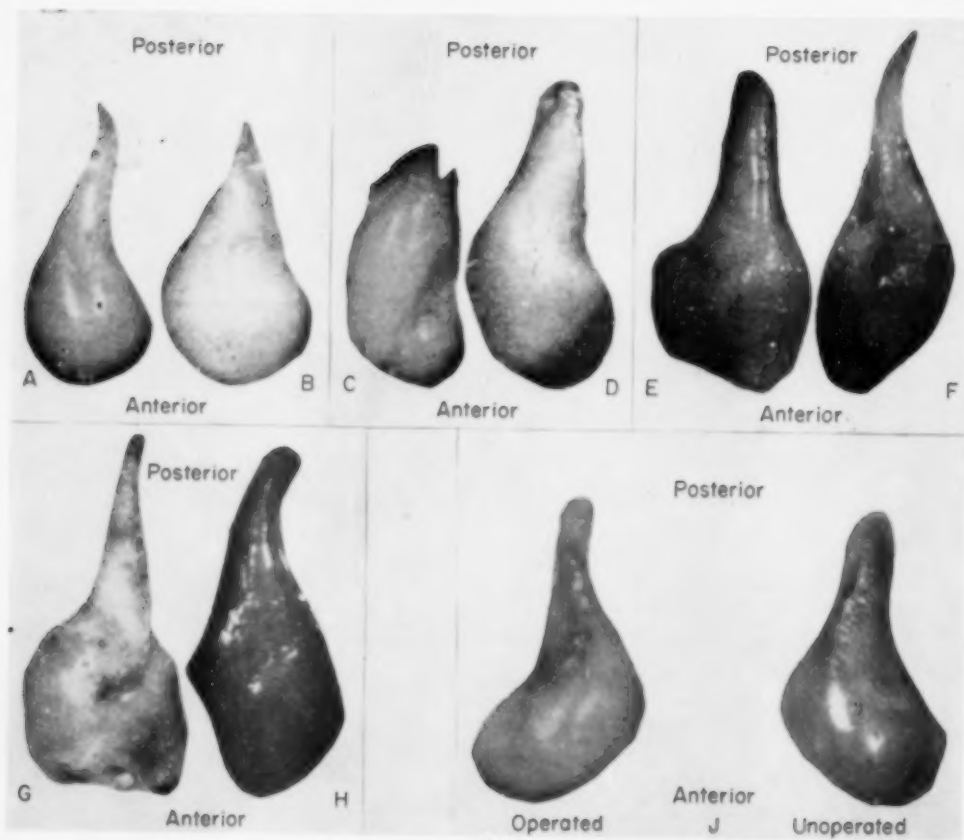


PLATE I



8

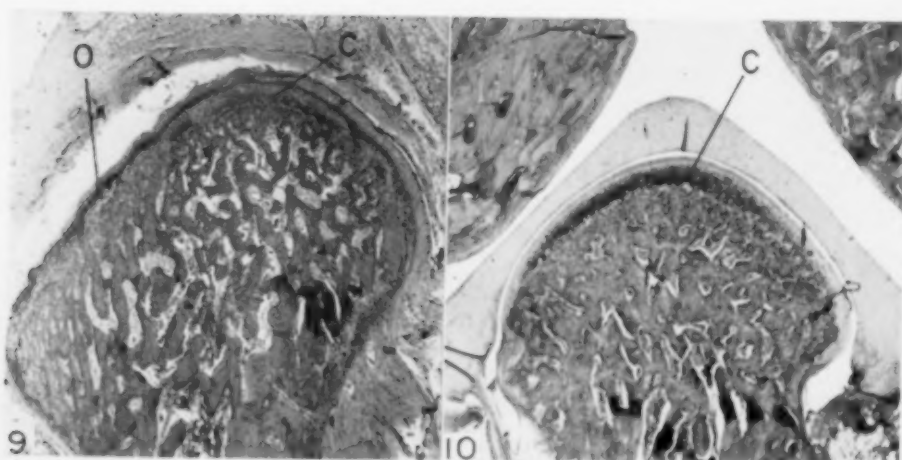


PLATE 2

PLATE 2

FIG. 8. Photographs of articular surfaces of mandibular condyles. $\times 4$.

- A and B following incision experiments (a) (PM 7 and 8); the condylar outline of the operated sides showed little change from the unoperated sides.
- C and D following division experiments (b); the condylar outline of the operated side C shows a reduction in length and a slight indentation; the disk in this joint (PM 5) shows extensive repair; the condyle D shows a marked widening and elongation; there was no repair of the disk in this joint (PM 6).
- E and F after excision of one quarter of disk (experiments c) (PM 9 and 10), the condyles show marked enlargement.
- G and H after excision of half of disk (experiments d) (PM 11 and 12), the operated sides show gross condylar enlargements and indentations; the disk in PM 11 (condyle G) was not recovered at postmortem.
- J a pair of mandibular condyles after control operation involving inferior and superior joint cavity (CO 12); the operated side differs little from the unoperated side—8 weeks after operation.

FIG. 9. Transverse section through mandibular joint after control operation involving both joint cavities; note flattening of articular surface and reduction of cartilage layer (C) at operation site (O). Haematoxylin and eosin. $\times 18$.

FIG. 10. Transverse section through unoperated mandibular joint showing intra-articular disk and regular distribution of cartilage layer (C) on condyle. Haematoxylin and eosin. $\times 18$.

AN INVESTIGATION INTO THE INTERDENTAL FORCES OCCURRING BETWEEN THE TEETH OF THE SAME ARCH DURING CLENCHING THE JAWS

J. W. OSBORN

Department of Dental Anatomy, Guy's Hospital, London Bridge, S.E.1

Abstract—A method of measuring interdental forces is described. Measurements were taken on ten subjects with the jaws relaxed and the jaws clenched. In all cases where a reading for the jaws relaxed was obtainable there was an increased force when the jaws were clenched. Those subjects with no caries and an excellent periodontal condition gave the highest readings. The possibility of a relationship between masticatory forces and drift of the teeth is indicated.

INTRODUCTION

AN ERUPTED tooth can be moved by forces acting on its crown and it may be that masticatory forces can contribute to this movement. When the masticatory muscles contract each tooth will be subjected to forces exerted through its antagonists in the opposing arch and the adjacent teeth in the same arch. The resultant of these forces may be split into directional components one of which acts through the contact surface between adjacent teeth in the same arch. The purpose of the investigation to be reported here is to measure the component of interdental force operating between teeth in the same arch during contraction of the masticatory muscles. It is known that teeth drift mesially (SICHER, 1960) and it is possible that this force may be responsible.

METHOD

Fig. 1 represents an occlusal view of two premolar teeth separated by the introduction of a length of orthodontic band between them. Separation of the teeth causes a recoil force R to be exerted on each side of the band. If the band is pulled with a force P in the direction indicated there is a frictional force F opposing withdrawal. When P is increased until movement just occurs then

$$P = F$$

$$F = 2\mu R$$

where μ is the coefficient of friction between tooth and band.

$$\therefore P = 2\mu R.$$

P can be measured with a spring balance and μ can be determined by experiment. Thus it is possible to calculate R , the recoil or interdental force.

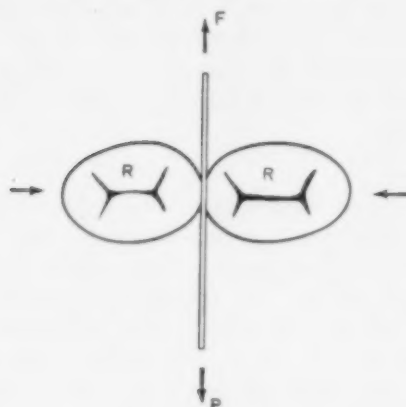


FIG. 1. Diagram of occlusal view of two premolars with band inserted between them showing the forces acting on the band during withdrawal.

Determination of μ

The determination of μ was made using teeth set up in plaster in a simple articulator. Known forces were applied through the contact surfaces which had been separated by stainless steel orthodontic band (2.5×0.1 mm). A spring balance (B) attached to this band made it possible to determine the forces necessary to remove the band and so to relate these to the applied forces. The slope of this curve is $1/2 \mu$. Fig. 2 shows the apparatus in diagrammatic form. In order to be sure that the

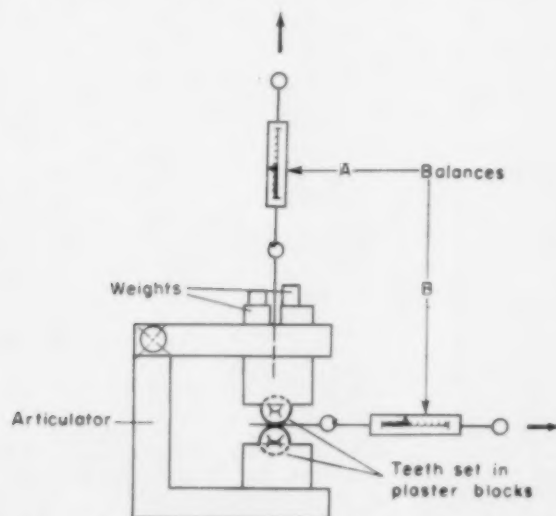


FIG. 2. Diagram of articulator with a tooth embedded in each of the upper and lower plaster blocks. The spring balances indicate how the measurements were taken to plot Fig. 3.

measurements of interdental force were accurate a spring balance (A) was attached vertically above the teeth and the actual values of the vertical forces acting through the contact surface were confirmed. Readings were taken with weights from 0.5 to 7 kg.

To simulate conditions *in vivo* as nearly as possible the band and tooth surfaces were moistened with saliva.

Experiments in vivo

The orthodontic band with spring balance attached was inserted between suitable contact surfaces in such a position that opposing arches could be occluded without interference. Force was gradually applied to the spring balance and the reading noted at which movement began. The direction of pull was in the plane of the contact surface of the teeth and at right angles to the occlusal plane. The subject was then asked to clench the teeth together as hard as possible and in this position a further reading was taken. Readings for the jaws relaxed and jaws clenched were taken as often as necessary in order to give reproducible values.

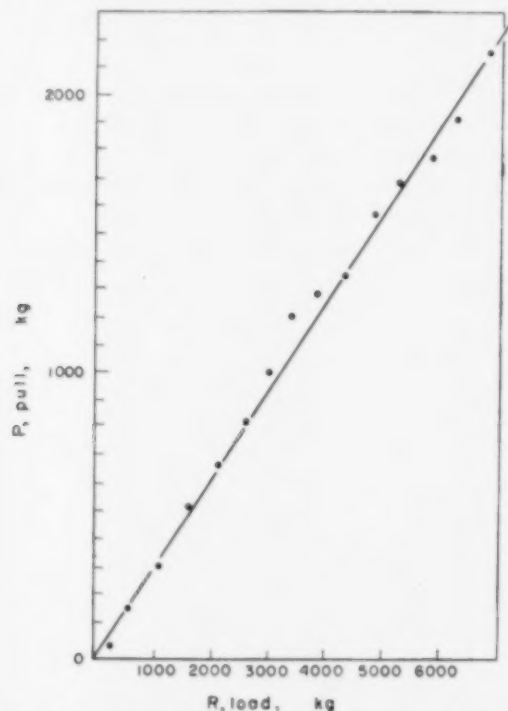


FIG. 3. Curve obtained from the *in vitro* measurements for 2μ , the coefficient of friction between a stainless steel band and the two teeth it is separating. The slope is $1/2\mu$.

RESULTS

From the experiment *in vitro* the value of $1/2\mu$ was calculated in order to determine R from the relationship

$$P = 2 \mu R$$

$$\text{or } R = 1/2 \mu P.$$

The value $1/2 \mu$ taken from Fig. 3 is 3.3.

$$\therefore R = 3.3 P.$$

Ten subjects were used in the experiment and as far as possible measurements were taken in all quadrants from canine—first premolar interspaces to first molar—second molar interspaces. In only two subjects (B and I) were erupted third molars present.

As an example of the accuracy of the measurements the full readings for one subject are given in Table 1.

TABLE 1. FIGURES OBTAINED FROM SUBJECT H

Upper right					Upper left				
Rel.	0	0	P.F.	450	Rel.	0	150	450	150
	0	0	P.F.	250		0	150	450	125
				350					125
Mean	0	0	—	350	Mean	0	140	450	140
Bit.	0	0	P.F.	900	Bit.	0	250	850	2070
	0	0	P.F.	2300		0	225	1000	1840
				2300			200	650	
								1000	
Mean	0	0	—	1830	Mean	0	225	880	1960
Diff.	0	0	—	1480	Diff.	0	85	430	1820
Rel.	200	150	600	1610	Rel.	300	400	400	600
	275	100	700	1380		350	375	300	500
	250								
Mean	240	125	650	1500	Mean	325	390	350	550
Bit.	450	150	1610	3220	Bit.	400	400	950	2530
	600	175	1380	3450		300	425	750	2300
	450								
Mean	500	160	1500	3340	Mean	350	410	850	2410
Diff.	260	35	850	1840	Diff.	25	20	500	1860
Lower right					Lower left				

Rel.=Relaxed jaws. Bit.=Jaws clenched. P.F.=Reading unobtainable. These figures are multiplied by $1/2 \mu = 3.3$ to obtain the figures in Fig. 11.

The histograms (Figs. 4-13) are divided into quadrants and show the results obtained in kilograms of interdental force in each interspace. The unshaded parts represent readings taken with the jaws relaxed; the shaded parts the increase in interdental force during clenching the teeth. The sum of the shaded and unshaded values is the actual reading obtained with the jaws clenched.

DISCUSSION

The readings obtained depended on the following variables:

1. *In the relaxed state*

The amount by which the teeth were displaced. This is dependent on the thickness of the band used and the resistance of the periodontal membrane to displacement of the teeth.

FIGS. 4-13. Histograms are divided into quadrants of the mouth.
Unshaded part: Readings with jaws relaxed.
Shaded part: Increase in interdental force during clenching the teeth.
Sum of unshaded and shaded parts: Actual reading obtained with jaws clenched.

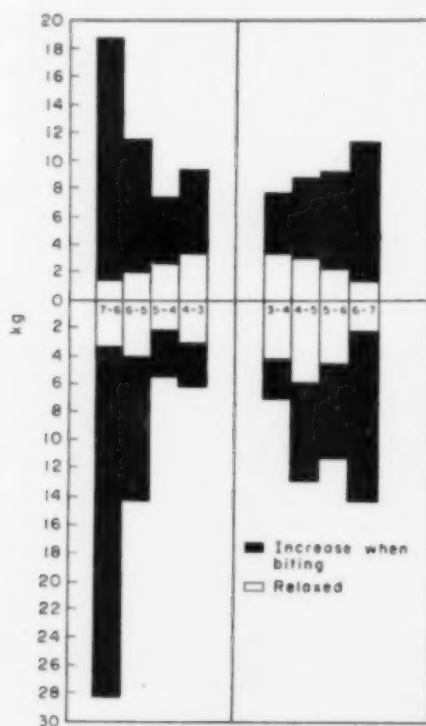


FIG. 4. Female. Age 19. Angle's Class I. \overline{E} retained and in occlusion. No caries. Periodontal condition excellent.

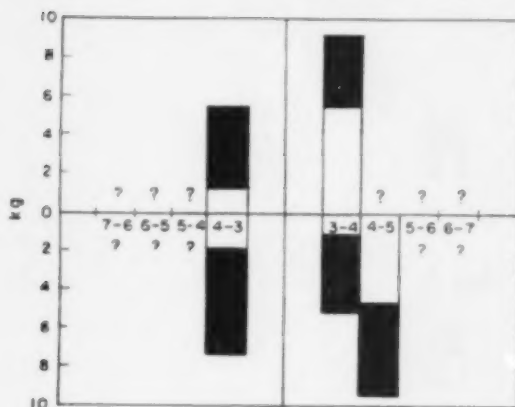


FIG. 5. Male. African. Age 23.
Angle's Class I. $\frac{8}{8}$ present. No caries.
Periodontal condition excellent. Remaining readings unobtainable due to very tight contact points.

FIG. 6. Female. Age 27. Angle's Class I. No caries. Periodontal condition excellent.

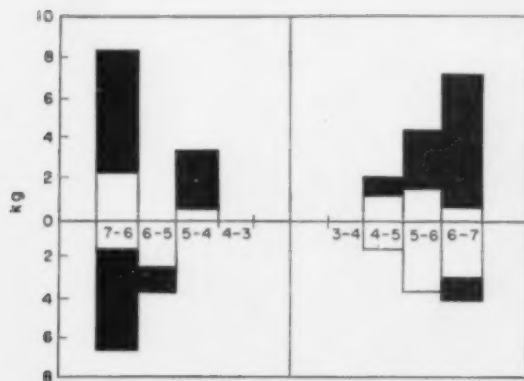
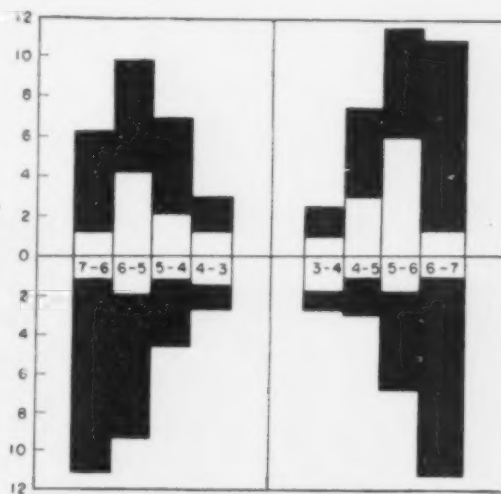


FIG. 7. Male. Age 19. Angle's Class I. $\frac{4}{4}$ instanding and out of occlusion. $\frac{43}{543}$ spaced. No caries. Periodontal condition excellent.

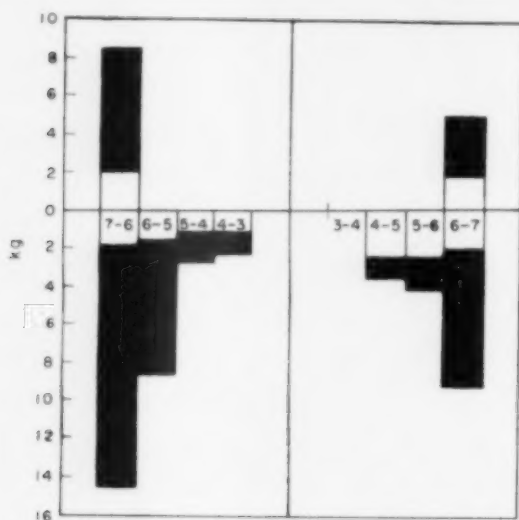


FIG. 8. Female. Age 19. Angle's Class II case treated by extraction of 5|5 and alignment of remaining teeth. Residual spacing 6543|3456. Few occlusal cavities. Periodontal condition excellent.

FIG. 9. Female. Age 20. Angle's Class I. Several interstitial fillings. Periodontal condition excellent.

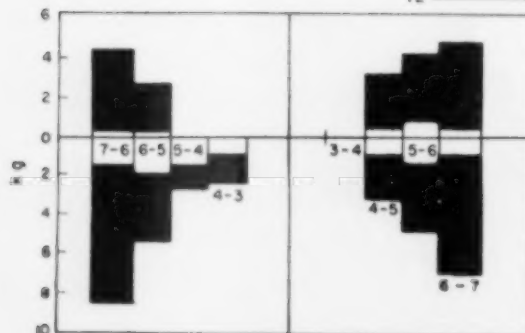
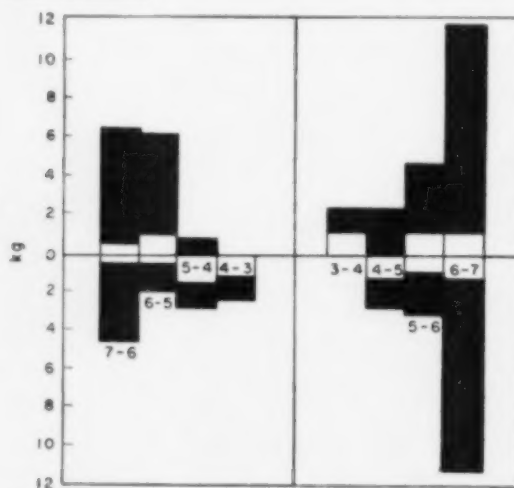


FIG. 10. Male. Age 18. Angle's Class I. Occlusal caries only. Periodontal condition good. Slack oral musculature.

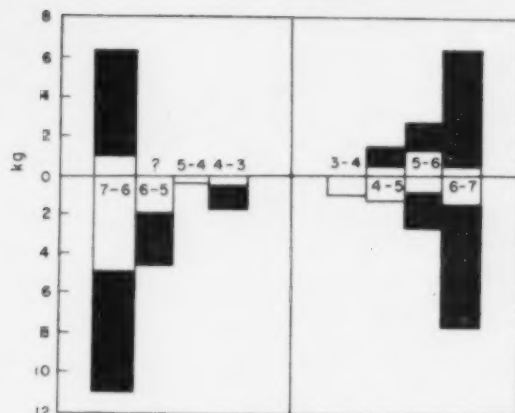


FIG. 11. Male. Age 16. Angle's Class I. $\overline{5}$ instanding and out of occlusion. $\overline{2}$ missing. $\overline{543}/\overline{34}$ spaced. $\overline{6}$ poor filling M.O. prevented reading. Extensive caries. Marginal gingivitis.

FIG. 12. Male. African. Age 25. Angle's Class I. $\overline{8}/\overline{8}$ present. Zinc oxide filling in $\overline{7}$. Extensive caries. Severe periodontitis simplex.

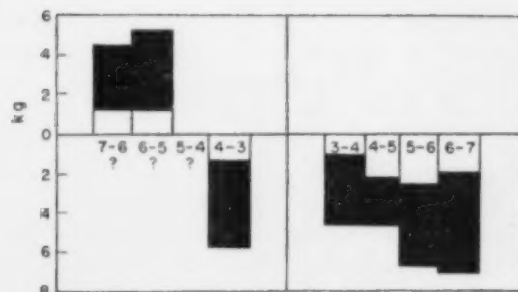
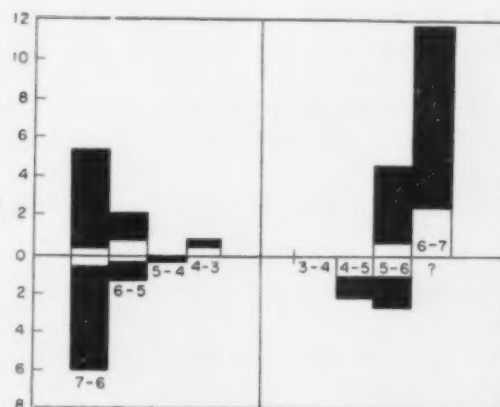


FIG. 13. Male. Age 16. Deep overbite. $\overline{4}/\overline{46}$ missing. $\overline{5}$ occludes mesial to $\overline{5}$ without touching $\overline{4}$. $\overline{7}$ occludes with $\overline{67}$. Upper right cheek teeth in buccal occlusion to lower right cheek teeth preventing any readings here. Moderate amount of caries. Periodontal condition good.

2. During clenching

- (a) The amount of displacement prior to clenching.
- (b) The magnitude and direction of force between opposing teeth which is dependent on:
 - (i) The angles of cuspal contact between opposing teeth.
 - (ii) The area of cuspal contact.
 - (iii) The magnitude and direction of force that the muscles of mastication give to the lower jaw.
- (c) The magnitude and direction of force between the condyles and their articular fossae.
- (d) Forces between adjacent teeth in the same arch.

These variables are interdependent.

The readings for the relaxed jaws are shown by the unshaded parts of the histograms. Those subjects with good periodontal conditions registered higher readings than those with some degree of gingivitis or periodontitis. The best dentitions (Figs. 4, 5 and 6) with no caries and no periodontal disease gave the highest and most symmetrical readings. Subject E, having a treated Class II dentition with a few occlusal fillings only and no periodontal disease gave symmetrical readings (Fig. 8). Except for subject D (Fig. 7) the remaining dentitions all suffered from either interstitial caries (Figs. 9-13), marginal gingivitis (Figs. 10 and 11), advanced periodontal disease (Fig. 12), or extremely irregular occlusion (Fig. 13). Readings obtained from these subjects either lacked symmetry or were much smaller than those for subjects A, B and C (Figs. 4, 5 and 6). Subject D (Fig. 7) with no caries or periodontal disease did not conform to the above pattern.

The increase in interdental force during clenching the teeth is shown on the histograms by the shaded areas. There was always an increase in this force when the jaws were clenched, and it could be brought about in three ways:

1. During clenching, by being pushed into the jaws, the teeth are squeezed together.
2. All the teeth are pushed mesially or all are pushed distally.
3. Some teeth are pushed mesially and some are pushed distally.

1. It is convenient here to consider the upper jaw. The crowns of the upper teeth are set on an arc (curve of Spee) mesiodistally. If each upper tooth is pushed into the jaw along its long axis then the $\overline{7|7}$ will move upwards and forwards and the $\overline{3|3}$ upwards and slightly backwards. This means that $\overline{7-3|3-7}$ will be squeezed together and account for the increased interdental forces during clenching. However, the forces on the lower teeth will be equal and opposite. That is, the lower teeth will be depressed and separated, e.g. $\overline{7|7}$ will be pushed downwards and backwards along their long axes. Since there was no reduction in interdental forces in the lower jaw during clenching then the first hypothesis is unlikely.

2 and 3. These can conveniently be discussed together. As an example the upper right quadrant of subject C (Fig. 6) can be considered. During clenching there was an increased force of $5\frac{1}{2}$ kg interdentally between $\overline{76|}$. Supposing that $\overline{7|}$ was not

pushed mesially, then the $5\frac{1}{2}$ kg must have been due to distal pressure from 6]. That is, there was an initial reduction in the interdental force between 65] of $5\frac{1}{2}$ kg. But a force of $4\frac{1}{2}$ kg was sufficient to separate 65] by the thickness of the band (0.1 mm) originally inserted here. (This is the measurement of the recoil force after insertion of the band with the jaws relaxed). Therefore, if, during clenching, 6] was pushed distally against 7] with a force of $5\frac{1}{2}$ kg a potential space of 0.1 mm must have resulted between 65]. But, in fact, during clenching there was an increased interdental force between 65] of 6 kg, therefore 5] must have been pushed back 0.1 mm against the resistance of its periodontal membrane, and with a further increase of 6 kg force. In the same way 4] and 3] must have been pushed back each 0.1 mm, leaving a space between 32]. But this did not occur and therefore, by *reductio ad absurdum*, the original hypothesis that 7] was not pushed mesially must be incorrect. In the same way it can be deduced that all the cheek teeth were subjected to a mesial force under the conditions of the experiment.

Further experiments are in progress to determine factors which influence these forces.

Acknowledgement—I am greatly indebted to Dr. D. J. ANDERSON for his help and encouragement in the preparation of this paper.

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A CLINICAL STUDY OF NON-ENDEMIC MOTTLING OF ENAMEL

D. JACKSON

The University of Leeds, Dental School and Hospital,
Leeds 1, England

Abstract—A study of the incidence and distribution of non-endemic mottling in 1040 12 year old school children and 298 adults, shows that such mottling may be due to a number of different causes. The relatively high incidence of mottling in the maxillary central incisor tooth, found in both children and adults, suggests a local cause which may be traumatic injury to the deciduous predecessors. Exanthematous fevers were the major cause of mottling in the first permanent molars but only a contributory factor in mottling in other teeth. In the remaining cases, the cause was considered to be systemic, operating at brief intervals throughout amelogenesis. The asymmetrical distribution of mottling is not considered inconsistent with this possibility. Low level fluorine intake cannot be dismissed as a possible cause.

INTRODUCTION

THE ONLY established fact concerning the aetiology of mottling in human enamel is that its incidence and severity are related to the concentration of fluorine in the drinking water. It is because of this that the term mottling is often used to imply dental fluorosis, whereas in fact the distribution of mottling, particularly in a non-endemic area, suggests that it may be brought about by a number of different mechanisms.

In his classification of mottled enamel DEAN (1934) gave the term "questionable" to that type of mottling in which the translucency of enamel was disturbed by white flecks or occasional white spots 1-2 mm in diameter. The term "questionable" was used by DEAN because he was doubtful whether this type of mottling was in any way connected with the more frank types of mottling seen in endemic areas and now known to be associated with fluorine. Although non-endemic mottling is quite common, it has never been thoroughly investigated and its cause has not been established. GOTTLIEB (1920) considered mottled enamel to be a mild variety of non-specific enamel hypoplasia, and if this hypothesis is accepted then any factor disturbing the process of calcification could be responsible for a whole range of enamel stigmata ranging from mild mottling to severe hypoplasia. Although many factors are known to be capable of producing enamel hypoplasia, only fluorine has been shown to be capable of producing both mottling and hypoplasia in human teeth. In attempting to assess the aetiological factor or factors producing non-endemic mottling, it would seem wise to reconsider those factors known to be capable of producing enamel hypoplasia.

Causes of enamel hypoplasia may be local or systemic. The two local causes are trauma and infection. BAUER (1946) has shown that abscessed deciduous teeth can seriously impair enamel calcification of the succedaneous teeth, a fact previously stated by TURNER (1912). The instances illustrated by BAUER were hypoplastic defects associated with abscesses in deciduous molar and incisor teeth and, although he considered only gross defects, it is at least possible that minor defects such as "questionable" mottling could have a similar aetiology. ANDERSEN (1942) gave the term "injury spots" to those defects of incisor enamel caused by trauma combined with infection of the antecedent deciduous teeth. Injury spots were characterized grossly by brownish stained depressions in those portions of the enamel calcifying at the time of the injury. "White spots" were described separately and trauma was not mentioned as an aetiological factor in this type of enamel defect. Nevertheless, the degree of damage would be related to the degree of trauma and it is conceivable that minor traumatic injuries could produce minor enamel defects, particularly in incisor teeth.

Among the known systemic factors capable of producing enamel hypoplasia are fluorine, rickets, hypoparathyroidism, exanthematous fevers, severe infections, and metabolic disturbances such as coeliac disease and acidosis. In a study of sixty non-leucic patients with enamel hypoplasia SARNAT and SCHOUR (1942) could find no definite cause but conceded that rickets, hypoparathyroidism and fluorine were possible causes. Exanthematous fevers were stated to be a less frequent cause than generally believed.

The critical level at which fluorine causes obvious or objectionable mottling is not necessarily the level below which fluorine ceases to be effective in producing any type of discernible mottling. DEAN was chiefly concerned with endemic mottling and since at the time of his investigation there would be few communities with the so-called critical level of fluorine, or above, in the drinking water, and many more communities with fluorine below this critical level, "questionable" mottling, because of its more universal occurrence, would be considered non-endemic in character. It is interesting to note that in seven communities with water supplies containing 0.4-2.5 p.p.m. fluorine DEAN, ARNOLD and ELVOVE (1942) recorded the mouth incidence of questionable mottling to range from 21.3 to 36.5 per cent, but in seven other communities with supplies containing <0.32 p.p.m. F the recorded mouth incidence ranged from 2.5 to 13.7 per cent. Although no comment was made about this seemingly marked division in the incidence of "questionable" mottling, the figures do suggest the possibility of fluorine as an aetiological agent even when the water supplies contain as little as 0.4 p.p.m. F. Although DEAN was cautious with respect to the aetiology of these white spots, SOGNAES (1941), studying this phenomenon in islanders of Tristan da Cunha, suggested that these enamel defects represent the mildest degree of dental fluorosis, the source of fluorine in that instance being food and not water. One cannot, therefore, as yet completely eliminate the possibility of fluorine as a factor in the aetiology of these white spots.

Clinical rickets in a civilized community is rare and might be dismissed as a major cause of mottling in such communities if it were not for the surprising findings

of FOLLIS, PARK and JACKSON (1952). These workers, in an attempt to determine the normal pattern of bone growth by studying the histology of developing bone in young children, found that up to 80 per cent of all children at 2 years of age had evidence of rickets. These children were all healthy and had died suddenly because of trauma or illness of only 1 or 2 days' duration; in many cases the previous nutrition was good, particularly with respect to vitamin D. The diagnosis of rickets was based upon the maturation cycle of the cartilage cell, which in rickets is interrupted and does not proceed to senescence and death. In bone, recovery from such a disturbance can occur, but in enamel any similar disturbance is irreversible. The ameloblast is very sensitive to disturbances of mineral metabolism and hence the observations of FOLLIS *et al.* (1952) are of particular interest in suggesting the possibility of a high order of enamel defects in apparently healthy young children. Another interesting feature of this work is that with severe infections of 10 days or more duration, new bone formation virtually stops. PARK (1954) states that "In the last analysis the action of severe infections on the bone cells probably has a nutritional basis".

Hypoparathyroidism and metabolic disturbances such as coeliac disease and acidosis are not likely to be major factors in mottled enamel aetiology, because of their comparative rarity relative to the high incidence of mottled enamel in non-endemic communities. The possibility remains, therefore, of the previously mentioned local factors, and of the systemic factors, fluorine, infections and sub-clinical rickets in the context of FOLLIS *et al.* (1952).

The first requisite in the study of a disease or anomaly is a full knowledge of its clinical incidence and distribution. In an attempt to contribute to such knowledge a study of the incidence and distribution of mottling was carried out on 1040 12-year-old school children and 298 adults (15-29 years), all residents of Leeds, a city with a water supply containing <0.5 p.p.m. F and an average content of 0.06 p.p.m. F.

Standards of diagnosis

DEAN (1934) classified mottled enamel into six grades according to severity: these were "questionable", "very mild", "mild", "moderate", "moderately severe", "severe". In non-endemic areas, most instances of mottling fall into DEAN's "questionable" grade and only a few into the "very mild" category and still fewer into the "mild" category. Clinical distinction between these grades presents the difficulty common to all subjective observations of diffuse limitations and particularly those of the minor grades. ZIMMERMANN (1954) in effect subdivided the "questionable" grade into "idiopathic" and "questionable" categories, somewhat confusing the original connotation of the grade "questionable". The term "idiopathic" was reserved for occasional oval opacities, usually occurring asymmetrically and usually only present in one or two teeth; in molar teeth the opacities of this grade were stated to involve cusp tips like snow-capped mountain peaks. The term "questionable" was then applied to that type of mottling which occurred as horizontal striations chronologically distributed and hence frequently symmetrical and occurring in several teeth. In molars, the opacities extended down the cuspal ridges. ZIMMERMANN considered his "idiopathic" grade to be "non-fluoride" and his

"questionable" grade to be "fluoride", thus presupposing aetiology. Even from ZIMMERMANN's description, clinical differentiation is often difficult, particularly in premolar and molar teeth and hence for the sake of clarity and without making assumptions in respect of aetiology the results of this investigation are presented without classification, any smooth enamel opacity being termed "mottled" regardless as to whether it was a fleck, streak or area and regardless as to its extent, and colour. The standards of diagnosis were precisely those used by DEAN (1934) and, whereas for reasons previously stated classification according to the various grades was not included in the final analysis, the type of mottling recorded was of the "questionable", "very mild" and "mild" types described by DEAN (1934).

The community index of DEAN (1942) depends on a clinical differentiation between the grades described and since such a distinction in a non-endemic area is sometimes diffuse, this index was not used.

Opportunity occurred to correlate the incidence of mottling with exanthematous fevers, records of which were available in most but not all of the school children examined. These data were obtained from the school medical history records.

FINDINGS

1. General mouth incidence (Table 1)

(a) *School children.* The general mouth incidence was approximately 34 per cent, constituting 29 per cent white mottling and 5 per cent brown mottling. The data did not suggest any sex difference in the general incidence.

TABLE 1. GENERAL MOUTH INCIDENCE OF MOTTLING
(Actual figures and percentages)
1040 12 year old Leeds children

	No.	White	Brown	None
Boys	508	131 25.8%	25 4.9%	352 69.3%
Girls	532	168 31.6%	30 5.6%	334 62.8%
Total	1040	299 28.7%	55 5.3%	686 65.9%

(b) *Adults.* No comparable figure can be given for mouth incidence in adults because of the undue loss of teeth.

2. Tooth incidence

(a) *School children* (Table 2, Fig. 1). The general tooth incidence was 3.1 per cent. For boys it was 2.9 per cent and for girls 3.4 per cent. A study of the incidence in respect of each tooth type is more illuminating. It will be noted that the maxillary central incisor (I_1) had an incidence of 10.3 per cent, three times the average tooth

incidence, and almost five times that of the mandibular central incisor. The mandibular teeth had a consistently lower incidence than those of the maxilla. Apart from the maxillary central incisor tooth, all the maxillary teeth were affected to a similar extent, ranging from 3.1 to 3.7 per cent.

TABLE 2. THE PERCENTAGE INCIDENCE OF MOTTLING IN MANDIBULAR AND MAXILLARY TOOTH TYPES OF 12 YEAR OLD CHILDREN

Tooth	Maxilla	Mandible
I ₁	10.3	2.2
I ₂	3.7	1.1
C	3.6	1.6
PM ₁	3.5	1.3
PM ₂	3.7	1.5
M ₁	3.6	2.8
M ₂	3.1	2.1

(b) *Adults* (Table 3). The distribution of mottling in the incisor teeth of 298 adults (15-29 years of age inclusive), shows a pattern almost identical with that found in school children. The incidence of mottling in the maxillary I₁ was 10.7 per cent as against 10.3 per cent in children. The corresponding figures for mandibular central

TABLE 3. THE PERCENTAGE INCIDENCE OF MOTTLING IN MANDIBULAR AND MAXILLARY CENTRAL AND LATERAL TEETH OF 298 ADULTS (Age range 15-29 years inclusive)

Tooth	Maxilla	Mandible
I ₁	10.7	2.4
I ₂	5.7	1.7

and lateral incisors were 2.4 and 1.7 per cent respectively as against 2.2 and 1.1 per cent in children. An incidence of 5.7 per cent was found in the maxillary lateral incisor tooth, being somewhat higher than the incidence found in the corresponding tooth of children (3.7 per cent).

3. Symmetry of distribution (Table 4)

Distribution symmetry could only be assessed when corresponding teeth either right or left, or upper or lower, were present. Thus a mottled upper tooth with a corresponding lower tooth also mottled, was counted as a symmetrical paired instance; if one was mottled and the other not, such was counted as a non-symmetrical paired instance.

An identical procedure was adopted when assessing symmetry between right and left. Only data for children are presented.

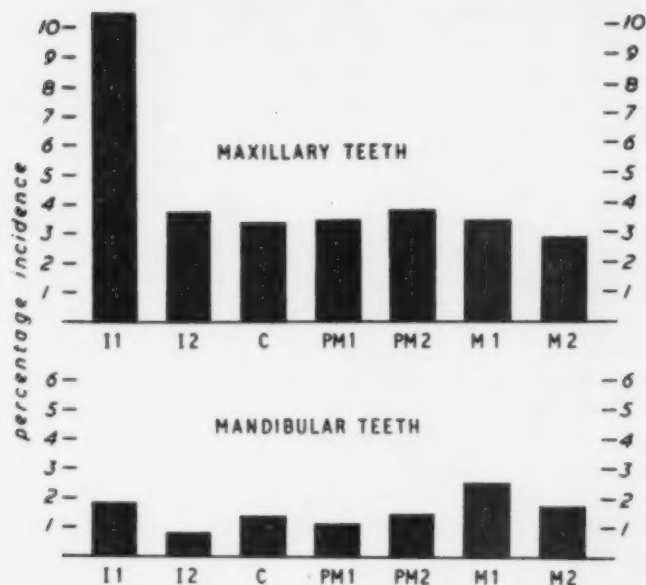


FIG. 1. The percentage incidence of mottling in mandibular and maxillary tooth types of 12 year old children.

(a) *Symmetry between right and left.* Out of 601 paired instances previously defined, only 167 or 27.8 per cent were found to be symmetrical with respect to mottling incidence. This low incidence of symmetry is surprising when the general tooth incidence for the right is almost identical to that for the left, either in the maxilla or in the mandible. For the upper right, upper left, lower right and lower left, the general tooth incidence figures were 4.5, 4.5, 1.9 and 1.6 per cent respectively.

TABLE 4. SYMMETRICAL DISTRIBUTION OF MOTTLING
Figures relate to the number of positively paired tooth instances
(12 year old children)

Right v. Left		Maxilla v. Mandible	
Symmetry	No symmetry	Symmetry	No symmetry
167	434	63	635

(b) *Symmetry between upper and lower.* Out of 698 paired instances only 63 or 9 per cent were found to be symmetrical with respect to the distribution of mottling. This low symmetry incidence is only to be expected because of the marked incidence difference between upper and lower teeth.

4. *Childhood illnesses and mottling* (Tables 5 a-g)

In correlating childhood illnesses to mottling, two criteria had to be applied to the data before they could be included. An illness could only be correlated with mottling in tooth types when (a) the illness occurred during the period of amelogenesis of the tooth type and when (b) all four of the respective and various tooth types (excluding third molars) were present. This meant that when considering the relationship between childhood illnesses and mottling in the first permanent molars for example, only the occurrence of such illnesses during the period of amelogenesis (0-3 years) was considered and even then only in those cases where all the first permanent molars were present. From the medical history, the illnesses considered were whooping cough, measles, diphtheria, scarlet fever, mumps, chicken pox and German measles.

If an illness occurred during the amelogenesis of a tooth then that tooth was said to have been subjected to potential risk. It sometimes happened that more than one illness occurred during the period of amelogenesis, and hence such a tooth could be said to have been subjected to multiple risks. The incidence figures, however, did not permit a separation of single and multiple risks. The periods of amelogenesis for tooth types were those stated by SCHOUR and MASSLER (1940). For incisors this was 0-5 years, for canines 0-7 years, for first premolars 1-6 years, for second premolars 2-7 years, for first permanent molars 0-3 years and for second permanent molars 2-8 years.

Because of the marked difference between central and lateral incisor teeth with respect to the incidence of mottling, these teeth were considered separately, although the periods of amelogenesis were taken to be broadly identical.

The data presented for this correlation are given in a series of four-fold tables; the corresponding figure for the chi-squared analysis is given when necessary. For $n=1$, χ^2 at P 5 per cent is 3.841 and at P 1 per cent is 6.635.

(a) *Central incisors* (Table 5 a). Of those subjected to potential risk, 5.7 per cent were mottled as against 5.8 per cent in those not subjected to risk. There is obviously no correlation between childhood illnesses and the incidence of mottling in the central teeth.

(b) *Lateral incisors* (Table 5 b). Of those subjected to potential risk, 2.6 per cent were mottled as against 1.5 per cent in those not subjected to risk. $\chi^2=3.622$. This value is barely significant at the 5 per cent level of probability.

(c) *Canines* (Table 5 c). Of those subjected to risk, 2.5 per cent were mottled as against 1.7 per cent in those not subjected to risk. $\chi^2=1.474$. This value is not significant at the 5 per cent level of probability.

(d) *First premolars* (Table 5 d). Of those subjected to risk 2.0 per cent were mottled as against 3.1 per cent in those not subjected to risk. Clearly, childhood illness could not be considered to be an aetiological factor of mottling in these teeth.

(e) *Second premolars* (Table 5 e). Of those subjected to risk 1.8 per cent were mottled as against 3.6 per cent in those not subjected to risk. As with the first premolars, childhood infection cannot be considered as an aetiological factor in these teeth.

(f) *First permanent molars* (Table 5 f). Of those subjected to risk, 3.3 per cent were mottled as against 1.1 per cent in those not subjected to risk. $\chi^2=9.473$. This result is highly significant, even at the 1 per cent level of probability, and the hypothesis that childhood infection has no influence on mottling in this instance can be rejected.

TABLE 5. THE RELATIONSHIP BETWEEN CHILDHOOD ILLNESSES AND THE OCCURRENCE OF MOTTLING

	Positive subjection to risk	No subjection	Total
(a) Central incisors (risk period 0-5 years)			
Mottling	174	56	230
No mottling	2844	909	3753
Total	3018	965	3983
Incidence of mottling (%)	5.765	5.803	5.774
(b) Lateral incisors (risk period 0-5 years) ($\chi^2=3.622$)			
Mottling	76	14	90
No mottling	2842	909	3751
Total	2918	923	3841
Incidence of mottling (%)	2.604	1.516	2.343
(c) Canines (risk period 0-7 years) ($\chi^2=1.474$)			
Mottling	69	12	81
No mottling	2655	676	3331
Total	2724	688	3412
Incidence of mottling (%)	2.533	1.744	2.373
(d) First premolars (risk period 1-6 years)			
Mottling	51	21	72
No mottling	2445	657	3102
Total	2496	678	3174
Incidence of mottling (%)	2.043	3.097	2.268
(e) Second premolars (risk period 2-7 years)			
Mottling	34	18	52
No mottling	1886	482	2368
Total	1920	500	2420
Incidence of mottling (%)	1.770	3.600	2.148
(f) First permanent molars (risk period 0-3 years) ($\chi^2=9.473$)			
Mottling	39	9	48
No mottling	1141	787	1928
Total	1180	796	1976
Incidence of mottling (%)	3.305	1.130	2.429
(g) Second permanent molars (risk period 2-8 years) ($\chi^2=0.204$)			
Mottling	55	13	68
No mottling	1833	499	2332
Total	1888	512	2400
Incidence of mottling (%)	2.913	2.539	2.833

(g) *Second permanent molars* (Table 5 g). Of those subjected to risk, 2.9 per cent were mottled as against 2.5 in those not subjected to risk. $\chi^2=0.204$. This value is not significant.

DISCUSSION

The mottling observed and referred to in this investigation is of the type which occurs in what is termed a "non-endemic" or "non-fluoride" area, and possesses an aetiology as yet unknown. It appears that this type of mottling has an incidence which can vary considerably from community to community. In Leeds school children, 34 per cent manifested mottling of one or more teeth. In a similar group of children in North Shields ($F<0.25$ p.p.m.), WEAVER (1944) found the incidence of mottling to be 27 per cent. FORREST (1956) reported the incidence of mottling in 12-14 year old school children in Saffron Walden and in West Surrey ($F=0.1-0.2$ p.p.m.) to be 63 and 60 per cent respectively. G. L. SLACK and F. E. LAWTON (personal communication) found the incidence of non-endemic mottling in 11 and 12 year old Liverpool school children ($F=0.09$ p.p.m.) to be 43.79 and 55.72 per cent respectively.

In the U.S.A. DEAN *et al.* (1942) gave incidence figures ranging from 2.5 to 43 per cent in communities which had water supplies containing less than 0.5 p.p.m. F. HURME (1949) gave an incidence figure of 83.5 per cent in a mixed group of people (13-30 years) said to have been born and reared in a non-endemic New England (U.S.A.) community. ZIMMERMANN (1954) in two Maryland (U.S.A.) counties ($F<0.2$ p.p.m.) found an incidence of 36 per cent in 12-14 year old children, and SELLMAN, SYRRIST and GUSTAFSON (1957) found the incidence in a similar group of children in Malmö (Sweden) ($F=0.5$ p.p.m.) to be 22 per cent. These incidence figures also include all the minor grades of mottling.

Whereas there are undoubted examiner differences with respect to the classification of mottling, it is unlikely that these would be so large as to account for the widely varying incidence figures which have been quoted. It appears, therefore, that community differences are both large and real.

It was previously suggested that the possible causes of non-endemic mottling were the local factors trauma and infection, and that the systemic factors were fluorine, exanthematous fevers and sub-clinical rickets. The possibility of these factors will now be discussed in the light of the evidence so far presented in this investigation.

The high incidence of mottling in the maxillary central incisors relative to the incidence found in other teeth, particularly to those of contemporaneous development, strongly suggests a local factor peculiar to these teeth. This local cause is unlikely to be infection resulting from caries, because the incidence of caries in the preceding deciduous tooth is extremely low relative to that found in the predecessors of the premolar teeth where mottling is very much less. The possibility remains, therefore, of trauma to the deciduous central incisors. Traumatic injury of the deciduous anterior teeth rarely results in fracture of the crown or root but commonly results in some loosening or displacement, which often completely resolves. It is, therefore, not possible in field studies to correlate the incidence of such injuries with the incidence of mottling, but in individual cases when histories are available,

such correlations can often be made. The developing crown of the central incisor is in very close proximity to the root of the deciduous predecessor and it is conceivable that the slightest apical movement or haemorrhage could interfere with the calcification of that part of the labial aspect of the crown developing at that particular time. The injury susceptibility order of permanent teeth is the upper central incisors, the upper lateral incisors, the lower central incisors and the lower lateral incisors, and it may be presumed that the injury susceptibility order of the deciduous anterior teeth is of the same pattern. This order is, in fact, the order of mottling incidence in the permanent incisors. It is suggested, therefore, that the high incidence of mottling in the maxillary central incisor is largely due to trauma of the preceding deciduous tooth and that a certain proportion of mottling in the remaining anterior teeth has this same aetiology.

It is not possible to associate trauma or infection with any other teeth. If this were so then the high incidence of infection in the deciduous molar teeth resulting in their early extraction (a possible traumatic factor) would assuredly be reflected in an outstandingly high premolar incidence. One would also expect the maxillary and mandibular teeth to be equally affected if this were true. Since neither of those circumstances occur, it may be concluded that the major cause of non-endemic mottling in all teeth except the maxillary central incisor is systemic.

There are, however, certain difficulties in reconciling the clinical distribution of non-endemic mottling with a systemic origin. One would expect a systemic factor to produce a chronological distribution, and a marked symmetry with respect to coronal location and actual incidence in teeth of contemporaneous development. In non-endemic mottling this type of distribution occurs with such infrequency that it could be considered statistically as purely a chance occurrence. The expectation of symmetry of both coronal location and of tooth type, depends, however, on the assumption that calcification in homologous teeth proceeds continuously with perfect synchrony. If, however, there were aphasic periods of calcification for "chronological" aspects of the same tooth, and if homologous teeth developed asynchronously then a systemic factor acting over a relatively short period could produce the asymmetrical and non-chronological type of mottling as seen in non-endemic areas. Phase asynchrony both with respect to coronal location and also to tooth types has indeed been observed in the distribution of mottling in the incisor teeth of experimental rats (JACKSON, 1956). The distribution of non-endemic mottling and indeed of certain enamel hypoplasias suggest that such asynchronies occur in the development of human teeth. A powerful inhibitor of calcification acting continuously over relatively long periods would produce chronological and symmetrical defects, by cancelling out any asynchrony of development.

It is, therefore, possible to reconcile non-endemic mottling with a systemic aetiology, and also to conclude that the factor or factors concerned operate at relatively brief intervals throughout the whole period of amelogenesis.

Such a factor could be the group of exanthematous fevers, but a positive correlation between this group factor and mottling was only found in respect of the first permanent molars. MASSLER and SCHOUR (1946) stated that there are three periods during

odontogenesis when there is an acute susceptibility to metabolic and cellular disturbances: these are 0-10 months, 2½ years and 5 years, the first being the most critical period. This exclusive correlation could tentatively be explained by the very rapid rate of amelogenesis of the first permanent molar (greater than in any other tooth) at a specific time of acute susceptibility. Thus a given factor would have a greater effect on the first permanent molars than on any other tooth.

It will be noticed that the incidence of mottling in those first permanent molars subjected to risk was three times that found in those not subjected to risk and hence it would appear that in a non-endemic community, exanthematous fevers are a major cause of mottling in these teeth. It would also appear that the major cause of mottling in other teeth (excluding the maxillary central incisor) is some other systemic factor. There is insufficient evidence to incriminate fluorine as this other factor, but it cannot be readily dismissed. The ingestion of fluorine from whatever source is likely to be a variable factor between individuals and at a low level of intake it is quite conceivable that only occasionally is the critical level with respect to mottling reached. Thus there could be an intermittent effective fluorine intake which could produce the non-endemic or asymmetrical type of mottling distribution. The question as to whether sub-clinical rickets is an aetiological factor must remain open but findings of FOLLIS *et al.* (1952) may be related to the susceptibility periods stated by MASSLER and SCHOUR (1946). So far no reference has been made to the distribution of non-endemic mottling in tooth types reported by other investigators. There are few comparable studies and reference can only be made to the investigations of HURME (1949) and ZIMMERMANN (1954). Neither of these investigators published figures for mottling in respect of individual teeth, and only an approximation can be assessed from their graphical presentations. It is quite clear, however, that the distribution and incidence figures found by these investigators differ not only with each other but also with those found in this investigation. For instance, HURME found mottling of all types to be present in approximately 80 per cent of all first permanent molar teeth: the comparable figure in ZIMMERMANN's investigation was 5 per cent, which is nearer to that found in the present investigation. For the upper central incisor, the incidence figure of HURME was approximately 10 per cent, whereas ZIMMERMANN's figure was 5 per cent.

In both ZIMMERMANN's and the present investigation the central incisors have a higher mottling incidence than lateral incisors and the upper central incisor has a higher incidence than the lower. The two investigations are in agreement in that maxillary premolars and molars have a similar incidence. There are, however, certain differences. ZIMMERMANN, for example, found the incidence in lower molars to be much higher than in upper molars, and the incidence in incisors to be much lower than that of the premolar incidence; there was no abnormally high incidence in the upper central incisor teeth.

It is apparent that more information is needed before definite conclusions can be reached concerning the aetiology of non-endemic mottling. There is every reason to believe that its aetiology is multiple, mainly systemic, and may vary between one locality and another.

Acknowledgements—The author wishes to acknowledge with thanks the grant made by Messrs. Thomas Hedley, Ltd. to the University of Leeds, by which this investigation was made possible. The author is also grateful for the co-operation of Mr. D. TAYLOR, Senior Dental Officer, Leeds Education Authority, and also for the assistance given by Miss A. COOPER.

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THE ULTRAHISTOLOGY OF BONE FORMATION

J. A. YAEGER

Department of Histology, College of Dentistry, University of Illinois,
808 South Wood Street, Chicago 12, Illinois, U.S.A.

Abstract—The palates of 1 day old mice were fixed in aqueous osmium tetroxide and some were stained with alcoholic phosphotungstic acid. Thin sections were stained and demineralized in aqueous phosphotungstic acid and/or stained in uranyl acetate, or were examined without staining.

In the differentiation of bone from mesenchyme, five phases could be discerned in the electron microscope: (1) The appearance of collagen fibrils among the pre-osteoblasts. (2) The aggregation of collagen fibrils into bundles. (3) The appearance of patches of inorganic particles, predominantly isometric in shape. After demineralization in aqueous phosphotungstic acid, condensed ground substance could be demonstrated between the fibril bundles in these patches. (4) The appearance of rodlet-shaped inorganic particles. (5) The coalescence of the isolated patches.

In the apposition of bone by osteoblasts upon the surface of bone spicules, most of the same phases occurred. The phase of isometric inorganic particles was not seen in this material, although ground substance condensation occurred in areas filled with inorganic rodlets. Isolated patches of matrix did not occur. Mineralized bone in the bodies of spicules was filled with inorganic rodlets. After demineralization, substantial areas of condensed ground substance, previously filled with rodlets, were disclosed.

INTRODUCTION

THE RELATIONSHIP between ground substance and collagen during the mineralization of bone matrix is controversial. The evidence implicating the ground substance polysaccharide-protein complexes as a crucial factor in mineralization includes the calcium-binding ability of mucopolysaccharides (BOYD and NEUMAN, 1951; FARBER, SCHUBERT and SCHUSTER, 1957) and the demonstration that agents which combine with mucopolysaccharides can inhibit mineralization *in vitro* (SOBEL, 1952; SOBEL and BURGER, 1954). In addition to the biochemical data, there is a substantial body of histochemical observation linking ground substance with the state of bone matrix mineralization. Changes in the reactivity to the Hotchkiss procedure of bone polysaccharides can be demonstrated in developing bone (COBB, 1953; HELLER-STEINBERG, 1951). Furthermore, Ca^{45} is bound to tissue sections predominantly in areas which stain metachromatically and contain organic sulphate (BELANGER, 1955). Finally metachromatic and Hotchkiss-positive material can be demonstrated in several mineralizing tissues (RUBIN and HOWARD, 1950).

An equally convincing array of information exists to implicate collagen as the principal organic matrix factor in bone mineralization. Demineralization of bone almost quantitatively liberates the epsilon-amino groups of matrix collagen (SOLOMONS and IRVING, 1958) and reprecipitated collagen can induce the formation of apatite in metastable calcium phosphate solutions (BACHRA, SOBEL and STANFORD, 1959;

GLIMCHER, HODGE and SCHMITT, 1957; STRATES, NEUMAN and LEVINSKAS, 1957). Demineralized bone matrix will also act as a seeding agent for apatite formation from metastable calcium phosphate solutions (GLIMCHER, 1959; LAMM and NEUMAN, 1958; STRATES and NEUMAN, 1958). Demineralized dentine has the same ability in nearly physiologic solutions, and it will not remineralize if the matrix carboxyl and epsilon-amino groups (presumably predominantly in collagen) are chemically blocked (SOLOMONS and NEUMAN, 1960). The histochemical evidence is more limited. Staining properties and birefringence of osteoid can be interpreted as indicating that collagen fibrils form adjacent to osteoblasts, disaggregate, and subsequently reaggregate immediately preceding mineralization (LOE, 1959). Finally, in thin sections of mineralizing bone studied with the electron microscope, some of the initial inorganic crystallites formed are on or within collagen fibrils (JACKSON, 1957; JACKSON and RANDALL, 1956; ROBINSON and CAMERON, 1956; SHELDON and ROBINSON, 1957).

The compromise view, that some combination of ground substance and collagen is necessary to induce or allow mineralization, is least well documented, however appealing. The *in vitro* mineralization of skin and tendon after removal of their ground substances, but not before, can be interpreted as indicating that the ground substances of these connective tissues prevent mineralization (GLIMCHER, 1959). On the other hand, complexes of collagen, fresh tendon or skin, and chondroitin sulphate will mineralize in metastable calcium phosphate solutions, while the fresh tissues or collagen alone mineralize less or not at all (BACHRA *et al.*, 1959; SOBEL and BURGER, 1954).

Electron microscopy, allowing us to view the morphologic changes occurring within and between collagen fibrils, should provide useful information concerning ground substance and collagen during bone mineralization. In several reports, mentioned above and described more fully in the discussion below, the initial appearance of inorganic crystallites on or within collagen fibrils during bone formation has been described. However, none of these reports excludes the occurrence of additional inorganic crystallites in the ground substance surrounding the fibril, or in the cementing substance surrounding the smaller units within the rather large fibrils which are illustrated. I am aware of no report in which ground substance changes are described in an electron microscopic investigation of thin sections of developing bone.

By using demineralized thin sections, changes in ground substance during bone formation could be observed, and will be described below. In addition, certain essential differences between the differentiation of bone matrix from mesenchyme in areas of endomembranous bone formation and the subsequent apposition of bone matrix upon a previously existing bone surface will be described.

MATERIALS AND METHODS

The palates of 1 day old mice were dissected free and split sagittally using razor blades. Efforts to cut smaller pieces of tissue failed. Apparently the spicules of bone present were displaced by the advancing razor's edge, crushing or tearing the tissue. The absence of bone in the middle of the palate eliminated this problem when splitting

them sagittally. The palate-halves were promptly immersed in chilled 1% buffered aqueous osmium tetroxide made hypertonic with sucrose (CAULFIELD, 1957). They were fixed for 30 min at 0°C and 90 min at room temperature, dehydrated at room temperature in ethanol solutions, and embedded in methacrylate (1-butyl/2-methyl). Some tissues were stained for 2 hr in 1% phosphotungstic acid dissolved in the 95% ethanol used during dehydration. Sections were cut with a diamond knife on a Porter-Blum microtome, floated on 15% acetone in water, flattened with xylene vapour (SATIR and PEACHEY, 1958), and mounted on formvar and carbon coated grids. Some sections were stained and demineralized for 1 hr with 10% phosphotungstic acid in water, stained with uranyl acetate, or treated with both reagents (WATSON, 1958). Electronmicrographs were prepared in an electron microscope with a resolution of 1.5 m μ .

For light microscopy, thick sections (1-2 μ) of palate halves fixed in chrome-osmium (DALTON, 1955) were stained with silver nitrate for bone mineral and counter-stained with fuchsin-alum haematoxylin (JENNINGS, FARQUHAR and MOON, 1959).

OBSERVATIONS

Demineralization by phosphotungstic acid

Staining sections with 10% phosphotungstic acid in water apparently demineralized them completely, revealing the underlying organic matrix (Figs. 4, 5, 8 and 10), while staining whole tissues with phosphotungstic acid in 95% ethanol had no apparent effect on inorganic particles (Figs. 7 and 9). However, where the shape of inorganic particles was of particular interest, tissues which had been fixed and stained with osmium only were used (Figs. 2 and 3).

Light microscopy of bone formation

In the palates of 1 day old mice, numerous rapidly growing spicules of bone could be seen with the light microscope. Along the broad surfaces of the spicules a layer of osteoblasts was usually found (A, Fig. 1). At the free edges of the spicules (D, Fig. 1) the intercellular substance of the adjacent soft tissue appeared homogeneous and the mesenchymal cells had lost their typical stellate form and were rounding. The rounded cells (pre-osteoblasts) near the edges of the spicules presumably are secreting the collagen and interfibrillar substance of the presumptive bone matrix. This matrix material aggregated, forming an optically homogeneous mass, and subsequently granules of mineral appeared which later coalesced with the body of the spicule. The surrounding osteoblasts (which have simultaneously differentiated from mesenchymal cells) begin the apposition of bone matrix upon this mineralized core of the spicule. This sequence of events occurred linearly in a section from the mesenchyme into the edge of a spicule.

Electron microscopy of the differentiation of bone from mesenchyme

In the electron microscope the intercellular spaces between the mesenchymal cells peripheral to a spicule of bone appeared empty. Apparently either the cells were shrunken or the intercellular material had been washed out. Moving towards the edge of the spicule the first hint of bone formation was the appearance of bundles

of parallel collagen fibrils. In this area no "empty" intercellular spaces could be seen, and the relationship between fibril bundles and cells could perhaps best be described as chaotic. Cell membranes could not be fully traced, and frequently what appeared to be cell organelles were immediately adjacent to fibril bundles. Because of the large pieces of tissue used, the fixation was not optimal, and cellular morphology of this material must be interpreted with caution. The diameter of the fibrils measured approximately 25–50 μm , and the cross-bands of the collagen were spaced by approximately 60–72 μm .

Nearer the edge of the spicule the collagen fibrils were less regularly arranged than those adjacent to pre-osteoblasts. Their diameters were essentially unchanged. Groups of from three or four to perhaps ten fibrils were aggregated into bundles in which the individual fibrils were still discernible as a scalloping at the periphery of the bundles (arrows, Fig. 2). These bundles measured approximately 150–500 μm in diameter, so that some of these bundles were within the limit of resolution of the light microscope. In long section it could be seen that the cross-bands of the fibrils were in register in a bundle (Figs. 4 and 5).

Just outside the edge of fully mineralized matrix, irregular patches (D, Fig. 1) often composed predominantly of opaque, isometric particles (Figs. 2 and 3) were seen. These particles measured approximately 2.5–5 μm in diameter. They were as electron opaque as the rod-shaped crystallites in the fully mineralized bone and were removed by demineralizing sections with phosphotungstic acid (Figs. 4 and 5). They were therefore interpreted as particles of inorganic material. Many rod-shaped crystallites measuring approximately 5×20 –50 μm were also seen in these patches. In other patches, the inorganic particles were nearly all rod-shaped. The infrequency of isometric particles in the latter patches suggests that the predominantly isometric particles in other areas were probably not rodlets viewed end-on.

After demineralization and staining in aqueous phosphotungstic acid, the patches containing inorganic particles were seen to consist of groups of fibril bundles obscured by a finely granular material (Figs. 4 and 5). The electron-opacity of this material approximated that of fragments of the cartilage of the nasal septum seen in these sections, and it was less than that of fully mineralized matrix (Figs. 7 and 9) or areas of partially mineralized matrix (Figs. 2 and 3). In some areas the cross-banding of the underlying collagen was clearly visible through this material (arrows, Fig. 5). Since this granular material persisted after sections were demineralized, it was interpreted as condensed ground substance rather than inorganic particles. The granules were not dense enough to allow precise measurement. The largest of them measured roughly 3.5 μm in diameter.

Electron microscopy of appositional bone formation

Moving on to the broad surface of the spicule, a layer of osteoblasts was encountered forming matrix on the calcified bone by surface apposition (A, Fig. 1). Most of the morphological phases of bone formation already described could be recognized, although they were crowded into a band measuring approximately 0.4–0.7 μm wide.

The osteoblasts were roughly oval in outline (Fig. 6). Even though the preservation of most cytoplasmic organelles was inadequate in this material, osteoblasts were nearly always separated from the bone matrix by a distinct and intact cell membrane (Figs. 6 and 7).

The uncalcified matrix adjacent to the osteoblasts contained collagen fibrils measuring approximately 25–50 m μ in diameter. Near the cell they occurred predominantly singly, further from the cell and nearer the mineralized matrix they occurred predominantly in bundles (Figs. 7 and 8). The interfibrillar material in this zone was either extracted or not stained by the preparative reagents. At the junction of calcified and uncalcified matrix, the collagen fibril bundles were suddenly completely masked by rod-shaped inorganic particles. In some areas only part of a fibril bundle was masked, and it was here evident that inorganic rodlets occurred within fibrils as well as between fibril bundles (arrow, Fig. 7). No isometric inorganic particles were seen in areas of bone apposition.

In sections stained and demineralized with aqueous phosphotungstic acid, the areas occupied by inorganic rodlets in bone formed by apposition contained condensed ground substance (G, Fig. 8) similar to that seen during the differentiation of bone matrix from mesenchyme.

Electron microscopy of mineralized bone

The more fully mineralized portions of the spicules demonstrated the typical ultrastructural pattern of calcified bone (Fig. 9). Nearly all of the organic matrix was masked by closely packed inorganic rodlets. Occasionally the outline of fibril bundles could be clearly discerned, and the inorganic rodlets were parallel to their surfaces. When similar sections were stained and demineralized with aqueous phosphotungstic acid, the cross-striated collagen fibril bundles were disclosed (Fig. 10). The spaces between bundles were filled with condensed ground substance which was generally finely granular, similar to that seen in areas of bone formation. The apparently vacuolar nature of the ground substance in some areas is discussed in a separate report. A comparison of mineralized and demineralized sections (Figs. 9 and 10) demonstrated that inorganic crystallites probably occurred both in the collagen fibril bundles and in the ground substance.

DISCUSSION

Phases of the differentiation of bone from mesenchyme

On the basis of the observations recorded above, the differentiation of bone matrix from mesenchyme in areas of endomembranous bone formation may be described as occurring in a sequence of five phases. (1) The secretion of collagen fibrils (or the secretion of molecular collagen and its prompt aggregation into fibrils). (2) The aggregation of groups of fibrils into bundles. (3) The condensation of ground substance between fibril bundles and the concomitant appearance of isometric inorganic particles, forming isolated patches. Condensation may be interpreted as any change in ground substance which allows more phosphotungstic acid and/or osmium binding, or reduces ground substance solubility in the preparative reagents.

(4) The aggregation of the isometric particles into rodlets, the definitive shape of bone inorganic particles. (5) The coalescence of the isolated patches to form a spicule of bone.

Phases of appositional bone formation

The apposition of bone matrix upon a bone surface occurred in essentially the same series of phases, with the following significant differences. (1) The phases occupied a much more limited area, so that they are not as clearly delimited as in the initial differentiation of bone matrix. (2) The phase in which isometric inorganic particles occurred was too brief to be observed in 1 day old mouse palate, although it has been described in other areas of bone apposition (ROBINSON and CAMERON, 1956; SHELDON and ROBINSON, 1957). Isolated patches of mineralized matrix do not occur since new matrix is deposited upon pre-existing matrix. (4) Although the present material was probably too young to demonstrate it, an additional phase should be added to appositional bone formation. In this phase the inorganic rodlets become distributed upon and within the collagen fibril bundles in a manner which emphasizes the collagen cross-banding (MOLNAR, 1959; ROBINSON and CAMERON, 1958; ROBINSON and WATSON, 1955; SCOTT and PEASE, 1956) and the collagen fibril bundles become more closely packed and parallel (ROBINSON and CAMERON, 1956; ROBINSON and WATSON, 1955).

Ground substance condensation

On the basis of the morphologic changes occurring during bone formation, the ground substance must be assigned a critical role in mineralization. The appearance of inorganic particles occurs nearly simultaneously with ground substance condensation. Since the morphologic change in the ground substance indicates little about the presumably concomitant chemical change, the alternative possibilities for the role of ground substance described in the introduction cannot be evaluated. But at least the ground substance cannot be omitted from an hypothesis accounting for the mechanism of bone formation and mineralization.

Cell surfaces

The preservation of cytoplasm in the material described here was not adequate to allow detailed evaluation of cell morphology. Some comment concerning cell surfaces may nevertheless be in order. The indefinite cell boundaries of pre-osteoblasts noted above have been described by others (JACKSON, 1957; JACKSON and RANDALL, 1956) and the intact cell membranes of mature osteoblasts forming matrix on bone surfaces are illustrated (but not described) in two previous reports (ROBINSON and CAMERON, 1958; SCOTT and PEASE, 1956). On the other hand, the cell membranes of mature osteoblasts have also been described as difficult to trace (SHELDON and ROBINSON, 1957).

Integration of previous electron microscopic investigations of bone formation

In studies of the initial differentiation of bone matrix around the cartilage anlage of fowl embryo bone, the appearance of collagen fibrils with diameters (12–50 m μ , 40 m μ) comparable to those described here have been reported (JACKSON, 1954,

1957). The aggregation of these fibrils into bundles of approximately the same diameter ($200\text{ }\mu$) as those described here and with cross-bands in register is evident in the micrographs of JACKSON (1957) and JACKSON and RANDALL (1956). In the latter two papers the appearance of material here interpreted as condensed ground substance was illustrated occurring before inorganic rodlets, but it was interpreted as the initial stage of mineralization. That some of such granules are not inorganic, at least in the present material, is established by their persistence in demineralized sections.

It is possible that the isometric inorganic particles seen in mouse palate were rodlets viewed end-on. This interpretation is unlikely to be correct since they were most frequent where rodlets were fewest and least frequent where rodlets were abundant. In both cases the rodlets were apparently randomly arranged. Furthermore, the appearance of isometric inorganic particles during the differentiation of bone matrix has been previously reported, and the particles described as being less than $100\text{ }\text{\AA}$ in diameter and yielding an apatite X-ray diffraction pattern (JACKSON, 1957; JACKSON and RANDALL, 1956). The inorganic particles were illustrated occurring in areas in which granular material masked the fibrils, again as in the present material, but with the difference in interpretation mentioned above.

The aggregation of the isometric particles into rodlets is illustrated and described by MOLNAR (1959) and strongly suggested by the work of ROBINSON and WATSON (1955). In both of these investigations the type of bone formation studied was appositional. Because of these reports, the isometric inorganic particle phase should probably be included in appositional bone formation, although not detected in 1 day old mouse palate. The isolated patches of inorganic particles described here have also been reported in descriptions of bone matrix differentiation (JACKSON, 1957; JACKSON and RANDALL, 1956).

Concerning appositional bone formation, collagen fibrils with diameters ($38\text{--}53\text{ }\mu$, $40\text{--}60\text{ }\mu$) compatible with those in the present material have been reported (ROBINSON and CAMERON, 1956, 1958; ROBINSON and WATSON, 1955). The aggregation of collagen fibrils into bundles had not been reported unless the pertinent figures in the papers describing differentiating matrix in fowl embryos (JACKSON, 1957; JACKSON and RANDALL, 1956) are of areas in which appositional bone formation had begun.

Although seen only in demineralized sections of the present material, the phase of ground substance condensation is visible in some of the published micrographs of mineralizing bone (ROBINSON and CAMERON, 1958; SHELDON and ROBINSON, 1957). This material was interpreted as the initial stage in mineralization, but persisted in mouse palate even after demineralization.

The isolated patches of inorganic particles described here in bone matrix differentiating from mesenchyme have also been described in appositional bone formation (ROBINSON and CAMERON, 1956, 1958; ROBINSON and WATSON, 1955; SCOTT and PEASE, 1956). When seen in the present material in areas of appositional bone formation, they could be interpreted as the result of nearly tangential sectioning through the irregular surface of mineralized matrix. The phase of appositional bone formation in which the inorganic rodlets come to emphasize the collagen periodicity

and the fibril bundles become closely packed and parallel was described in the same reports. Probably the age of the animals used in the present study precluded the occurrence of such a phase.

One observation demands separate consideration. In thin sections of frozen-dried, unstained bone, a zone of noncrystalline or microcrystalline (less than 10 Å in diameter) inorganic material has been described between the uncalcified and fully calcified matrix (MOLNAR, 1959). Freezing and drying without staining is certainly less likely than aqueous osmium fixation to remove or alter such a phase in mineralization. The theoretical validity and potential significance of this observation demands that an effort be made to verify it in material also examined after osmium fixation, so that this phase can be placed in its proper relationship with those described above.

Acknowledgements—It is a pleasure to acknowledge my indebtedness to Mrs. ALINA SKRUPSKELIS for technical assistance, and to Dr. JOHN MARBARGER, Miss IRENA KAIRYS and Mr. WILLIAM KADETZ of the University of Illinois Aeromedical Laboratory for providing and maintaining the electron microscopes used in this study.

This investigation was supported by a P.H.S. Research Grant (A-3220) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

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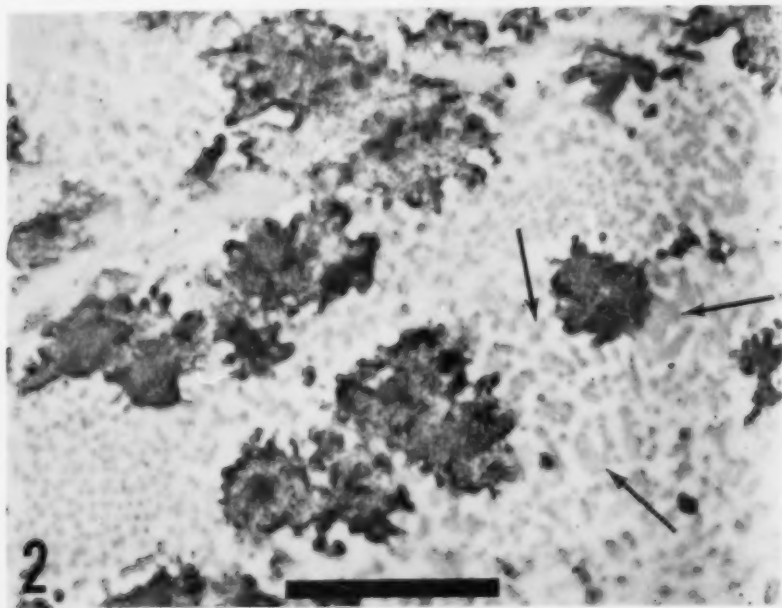
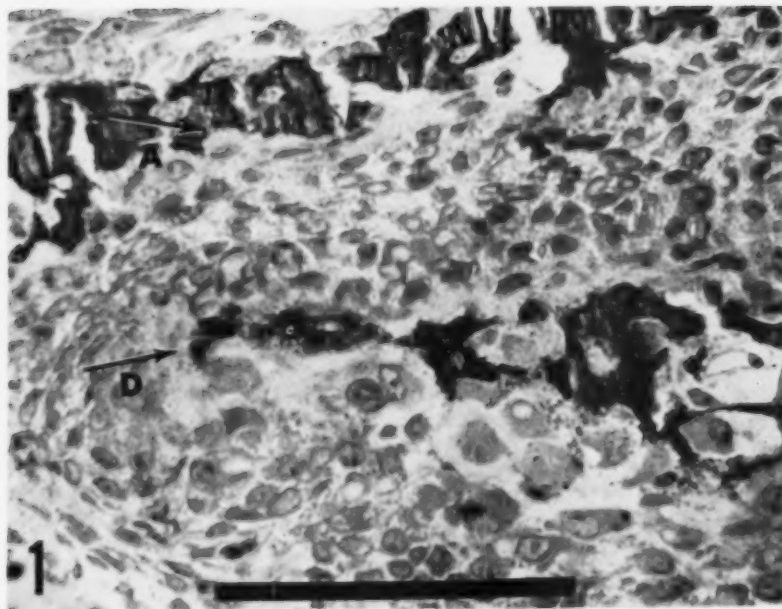
PLATE I

Both the figures are micrographs of sections of 1 day old mouse palate

FIG. 1. Light micrograph of tissue fixed in Dalton's chrome-osmium, sectioned at $1\ \mu$ and stained with fuchsin-alum haematoxylin and silver nitrate. The initial differentiation of bone matrix from mesenchyme is indicated at D, and osteoblastic apposition of matrix upon pre-existing bone is indicated at A. Bar at bottom represents 0.1 mm. $\times 510$.

FIG. 2. Electron micrograph of bone matrix differentiation similar to that at D in Fig. 1. Tissue fixed in buffered osmium tetroxide. Several patches of inorganic particles are included. The arrows indicate bundles of collagen fibrils. Bar at bottom represents $1\ \mu$. $\times 30,700$.

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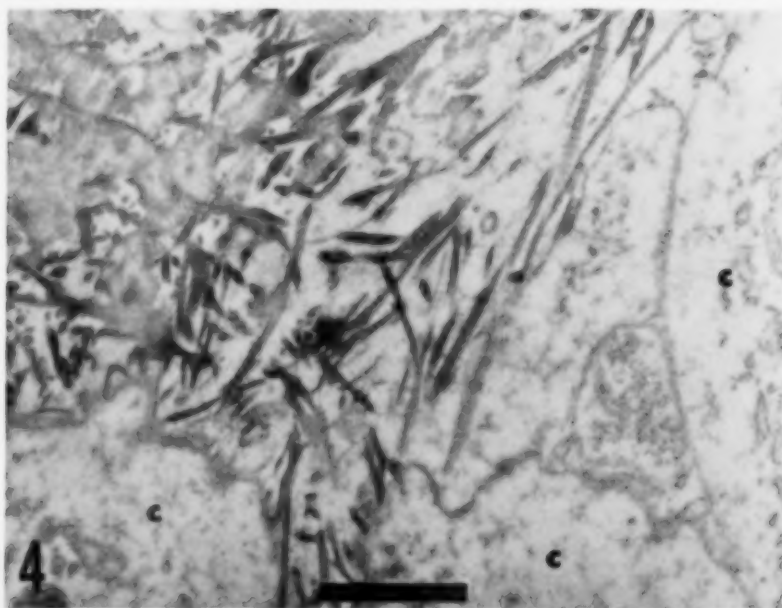
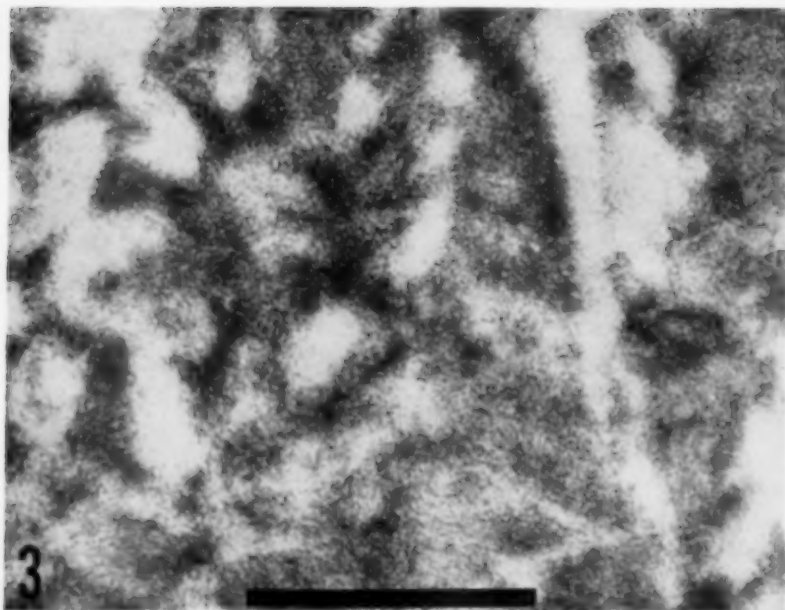


PLATE 2

PLATE 2

Both the figures are micrographs of sections of 1 day old mouse palate

FIG. 3. Electron micrograph of a patch of inorganic particles similar to those in Fig. 2. The tissue was fixed in buffered osmium tetroxide. Note that most of the particles are isometric. Bar at bottom represents 0.5μ . $\times 82,700$.

FIG. 4. Electron micrograph of an area similar to that of Fig. 2. The tissue was fixed in buffered osmium tetroxide, and stained in alcoholic phosphotungstic acid. The section was demineralized and stained in aqueous phosphotungstic acid and stained with uranyl acetate. The cytoplasm of pre-osteoblasts is evident at C. Condensed ground substance partially makes the collagen fibrils at the upper left. Bar at bottom represents 1μ . $\times 21,000$.

PLATE 3

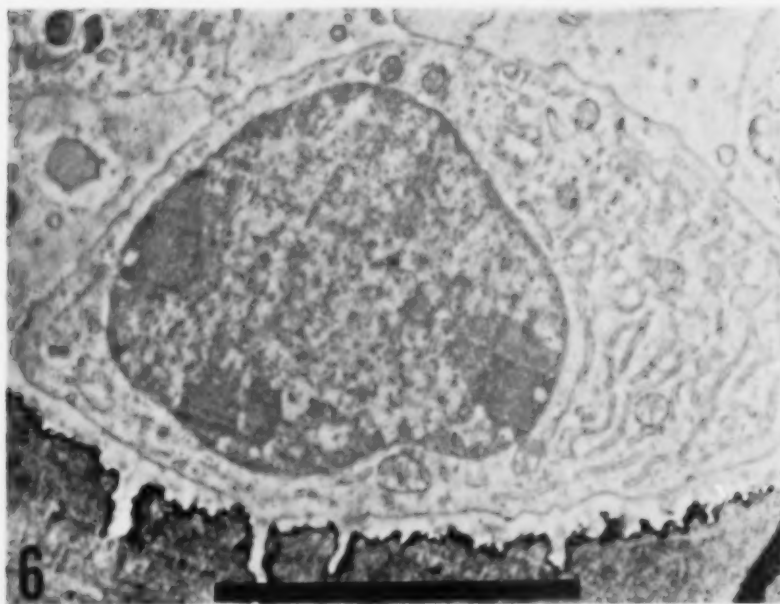
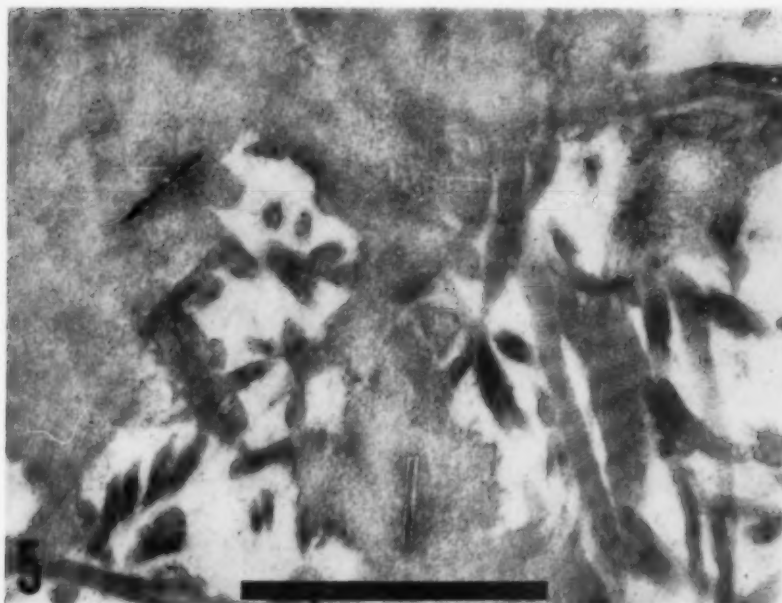
Both the figures are micrographs of sections of 1 day old mouse palate

FIG. 5. Higher magnification electron micrograph of condensed ground substance in an area from Fig. 4. The arrow indicates collagen cross-banding. Bar at bottom represents 0.5μ . $\times 87,000$.

FIG. 6. Electron micrograph of an osteoblast in an area similar to that at A in Fig. 1. The tissue was fixed in Dalton's chrome-osmium. Mineralized bone is evident at the bottom. Bar at bottom represents 5μ . $\times 10,400$.

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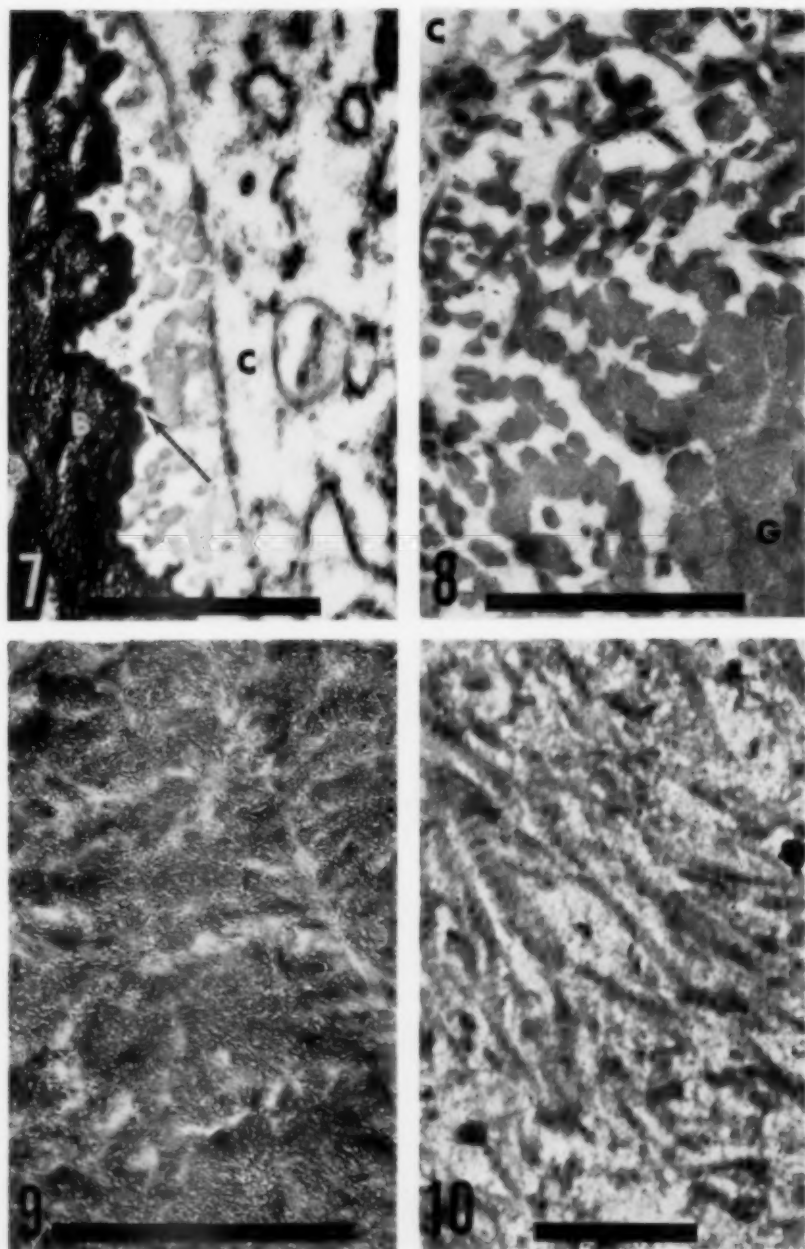


PLATE 4

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PLATE 4

All of the figures are micrographs of sections of 1 day old mouse palate

FIG. 7. Electron micrograph of an area of appositional bone formation similar to that between the osteoblast and calcified bone in Fig. 6. The tissue was fixed in buffered osmium tetroxide and stained in alcoholic phosphotungstic acid. Osteoblast cytoplasm is at C, calcified bone at B. The arrow indicates three incompletely mineralized bundles of collagen fibrils. Bar at bottom represents 1μ . $\times 33,900$.

FIG. 8. Electron micrograph of an area similar to that of Fig. 7. The tissue was prepared as in Fig. 7, and the section demineralized and stained in aqueous phosphotungstic acid and stained with uranyl acetate. Osteoblast cytoplasm is at C, condensed ground substance of previously mineralized bone at G. Bar at bottom represents 0.5μ . $\times 73,900$.

FIG. 9. Electron micrograph of mineralized bone. The tissue was fixed in buffered osmium tetroxide and stained with alcoholic phosphotungstic acid. Note that the inorganic particles are rodlets. Bar at bottom represents 1μ . $\times 43,200$.

FIG. 10. Electron micrograph of an area similar to that of Fig. 9. The tissue was prepared as for Fig. 9, and the section was demineralized and stained in aqueous phosphotungstic acid. Note the areas of condensed ground substance between collagen fibril bundles. Bar at bottom represents 1μ . $\times 23,200$.

THE APPARENT FIRST DISSOCIATION CONSTANT (pK'_1) OF CARBONIC ACID IN SALIVA

S. WAH LEUNG*

University of Pittsburgh School of Dentistry,
Pittsburgh 13, Pennsylvania, U.S.A.

Abstract—The wide discrepancy which we had previously reported between the pK'_1 of carbonic acid in saliva as calculated from the Henderson-Hasselbalch equation and as calculated from the ionic strength of saliva led us to re-examine this problem to try and account for the difference. From purely theoretical considerations we postulated that the discrepancy may be due to the fact that not all of the bound- CO_2 in saliva was in the form of bicarbonate but that, in fact, part of it was present in the carbamino form. This hypothesis was tested experimentally and found to be valid. When the amount of carbamino CO_2 in saliva (about 25 per cent of the total CO_2) was taken into consideration in calculating the pK'_1 by the H-H equation, the result (6.28) was almost identical with the anticipated value of 6.27.

INTRODUCTION

THE HENDERSON-HASSELBALCH equation, when applied to the bicarbonate : carbonic acid buffer pair, defines the pH of the buffer solution as:

$$pH = pK'_1 + \log \frac{[BHCO_3]}{[H_2CO_3]} \quad (1)$$

The term pK'_1 is the negative logarithm of the apparent first dissociation constant of carbonic acid, and $[BHCO_3]$ the concentration of bicarbonate. If the pH and the ratio of bicarbonate:carbonic acid of the buffer solution are known, the pK'_1 should be readily calculated.

The principal buffer system of saliva has been shown to be the bicarbonate: carbonic acid pair (LEUNG, 1951). If all of the CO_2 in saliva is present only as bicarbonate and carbonic acid, then:

$$\text{Total } CO_2 = BHCO_3 + H_2CO_3 \quad (2)$$

When the total CO_2 of saliva and either of the other two terms are known, the third may thus be calculated.

Standard methods are available for the direct determination of total CO_2 . If the CO_2 tension (pCO_2) is known, the H_2CO_3 may be calculated by the formula:

$$H_2CO_3 \text{ (vol. \%)} = \frac{\alpha pCO_2 \times 100}{760} \quad (3)$$

provided α , the solubility coefficient of CO_2 in the solution under consideration, is also known.

* Present address: University of California School of Dentistry, Los Angeles 24, California, U.S.A.

In a previous study in which the pK'_1 of carbonic acid in saliva was determined (LEUNG, 1950) we had assumed that all of the CO_2 in saliva was in the form of either bicarbonate or H_2CO_3 . The latter was calculated by equation (3), in which the pCO_2 was experimentally determined, and α for saliva assumed to be similar to that reported for lymph, namely, 0.528 (VAN SLYKE *et al.*, 1928). The bicarbonate concentration was thus obtained by subtracting the calculated H_2CO_3 from the measured total CO_2 . By substituting these values in equation (1) the pK'_1 was calculated for fifty-one samples of human, paraffin stimulated, mixed saliva, over a pCO_2 range of 0 mmHg to 150 mmHg. The average pK'_1 for these fifty-one saliva samples was found to be only 6.00, which is much lower than we had expected on the basis of the known physico-chemical behaviour of carbonic acid.

According to SENDROY and HASTINGS (1925) the pK'_1 of carbonic acid is related to the ionic concentration of the solution (μ) as:

$$pK'_1 = 6.33 - 0.5 \sqrt{\mu} \quad (4)$$

SCHMIDT-NIELSEN (1946) measured the electroconductivity of twenty-five samples of human mixed saliva and found the average to be equivalent to that of a 27.5 mM NaCl solution. On this basis, the pK'_1 , as calculated by equation (4), should be 6.27, and not 6.00. Efforts to account for this discrepancy between the theoretical and the measured pK'_1 constitute the basis for this paper.

Assuming that our experimentally determined pK'_1 to be wrong, and that the theoretical value of 6.27 to be more nearly correct, the error may be due to having either too small a value for H_2CO_3 or too large a one for $BHCO_3$.

Possible error due to H_2CO_3

In calculating the H_2CO_3 by equation (3) it was assumed that α for saliva was the same as for lymph. Since our original work in 1949, SAND (1949) has reported a value of α for CO_2 in saliva of 0.562, as contrasted to the lower value of 0.528 which we had previously used. When SAND's value for α was used to re-calculate our original data for H_2CO_3 the pK'_1 was raised only very slightly, from 6.00 to 6.03. This is still much lower than the theoretical 6.27. Thus, the discrepancy could not be accounted for on the basis of using an erroneous value for α . The remaining possibility would be that the $BHCO_3$ values used were consistently higher than the true values.

Possible error due to $BHCO_3$

As was pointed out above, in calculating the $BHCO_3$ we had assumed that all of the CO_2 in saliva was present only as H_2CO_3 and $BHCO_3$, namely, that all of the so-called bound- CO_2 was present as bicarbonate. However, it has been shown by HENRIQUES (1928), MELDRUM and ROUGHTON (1933), MARGARIA and GREEN (1933), and others, that in solutions containing free amino groups ($-NH_2$) the amino groups will combine with CO_2 to form carbamate or carbamino compounds, the amount thus formed being a function of the pH. In view of the fact that a complex fluid such as saliva contains a variety of compounds which, under appropriate conditions, can contribute $-NH_2$ groups for combination with CO_2 , it would be logical to assume

that not all of the bound- CO_2 in saliva is necessarily in the form of bicarbonate, and that significant amounts of it may be in the form of carbamate. Thus the "true" bicarbonate concentration would be less than we had previously assumed, and the pK'_1 would be higher than formerly reported. This possibility was tested experimentally as described below.

EXPERIMENTAL PROCEDURE

Mid-morning samples of paraffin stimulated mixed saliva were collected from four male dental students. The saliva was deposited under a layer of mineral oil in an iced test-tube. Immediately following collection the sample was aspirated into a 30 ml hypodermic syringe without exposing the sample to air, and the syringe sealed by inserting the end of the hypodermic needle into a rubber stopper. All dead space in the syringe had been filled with liquid paraffin prior to aspirating the sample. The samples were stored in this fashion in the refrigerator until used. All analyses were usually completed on the same day.

The following analyses were done on each sample: pH, with the Leeds and Northrup pH indicator No. 7664; total CO_2 by the manometric method of VAN SLYKE and NEILL (1924); pCO_2 by the method of RILEY, PROEMMEL and FRANKE (1945); and "carbamate CO_2 " by the method of FERGUSON and ROUGHTON (1934). From the analytical data the following were calculated: H_2CO_3 by equation (3), using the solubility coefficient of 0.562 as given by SAND; BHCO_3 by taking the difference between the total CO_2 and the sum of H_2CO_3 and "carbamate CO_2 "; pK'_1 by equation (1). Average pK'_1 's were determined by first converting the individual pK'_1 's to the corresponding K'_1 's.

RESULTS AND DISCUSSION

A total of 102 saliva samples were collected from the four subjects. The data are summarized in Table 1.

TABLE 1. CARBON DIOXIDE CONTENT AND DISSOCIATION CONSTANT OF CARBONIC ACID IN HUMAN SALIVA

Subject	N	Total CO_2 (mM/l)	Carbamino CO_2 (mM/l)	BHCO_3 (mM/l)	H_2CO_3 (mM/l)	pCO_2 (mmHg)	pH	K'_1 ($\times 10^{-7}$)	pK'_1
S.M.	16	$16.22 \pm 1.39^*$	3.00 ± 0.63	12.32 ± 1.94	1.03 ± 0.23	30.85 ± 6.85	7.31	5.88	6.23
A.J.	37	12.51 ± 2.38	4.05 ± 1.35	7.46 ± 2.66	1.10 ± 0.23	32.90 ± 7.28	7.14	4.92	6.31
D.J.	26	12.09 ± 2.13	2.76 ± 0.88	8.36 ± 2.70	0.96 ± 0.19	28.79 ± 5.62	7.25	4.95	6.31
C.P.	23	20.30 ± 1.55	3.84 ± 1.22	16.42 ± 1.45	0.93 ± 0.19	27.83 ± 5.43	7.53	5.87	6.23
Total	102	15.39 ± 4.89	3.50 ± 1.23	10.95 ± 4.95	1.01 ± 0.36	30.40 ± 6.69	7.31	5.20	6.28

* Standard deviation.

The total CO_2 and the bicarbonate contents showed considerable variation among the four subjects, as well as within the same subject. The pCO_2 and, therefore, the H_2CO_3 are more constant. However, the pCO_2 values for our subjects are much

lower than has been reported by others (HENDERSON and STEHLE, 1919; SAND, 1949). It is likely that the present data do not reflect the true pCO_2 of saliva as secreted, since no effort was made to prevent the loss of CO_2 upon exposure of the saliva to the oral cavity. It is quite possible that the pCO_2 would have been higher had the saliva been collected by cannulation of the ducts rather than by free flow into the oral cavity. However, since the various fractions of CO_2 are presumably in equilibrium with one another, changes in the pCO_2 should affect all fractions to a proportionate degree.

The actual values for total CO_2 , and, therefore, for the other CO_2 fractions as well, should probably be slightly higher than reported here, since some of the CO_2 may have been absorbed by the mineral oil. Here again, however, one should expect the various CO_2 fractions to be affected proportionately, assuming they are in equilibrium with each other and with that dissolved in the mineral oil.

The carbamate CO_2 constituted from 17 to 32 per cent of the total CO_2 and from 17 to 35 per cent of the bound- CO_2 of saliva. The average values for all subjects are, respectively, 23 per cent and 24 per cent. Thus, approximately one-quarter of the CO_2 in saliva is bound to $-NH_2$ groups, and only about 75 per cent of the bound- CO_2 is bicarbonate.

Of particular interest to our present study are the pK'_1 values. As can be seen from the last column in Table 1, these values are in close agreement with that of 6.27, calculated from equation (4) using the ionic strength as reported by SCHMIDT-NIELSEN (1946). While there was some variation in this value among the different subjects, the average value for the 102 saliva samples was 6.28, a difference of only 0.01 unit from the theoretical value. The close agreement between the theoretical and the experimentally determined pK'_1 's strongly suggests that carbamino compounds are of quantitative importance in the buffering capacity of saliva.

Acknowledgements—This investigation was supported by a research grant, D-189, from the National Institute of Dental Research, Public Health Service. Part of the results were reported at the Symposium of the 25th Year Celebration of the University of Rochester Dental Research Fellowship, Rochester, N.Y., 8 October 1955, and at the 35th General Meeting of the International Association for Dental Research, 21–24 March 1957.

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A HISTOCHEMICAL STUDY OF THE INTERTUBULAR AND PERITUBULAR MATRICES IN NORMAL HUMAN DENTINE

N. B. B. SYMONS

Dental School, University of St. Andrews, Dundee, Scotland

Abstract—Serial ground sections of sound, recently erupted human teeth were stained with 0.0005 M methylene blue, 0.01–0.1% toluidine blue, 0.05–0.5% alcian blue at pH 2.6, 3.6 and 7.0, and with the periodic acid–Schiff method.

With methylene blue at pH 2.6 and 3.6, a zone of dentine about 20–25 μ wide, immediately adjacent to the predentine, was stained. The staining was in the intertubular matrix which also stained intensely with alcian blue and the PAS method and showed metachromasia at pH 3.6 with toluidine blue. This zone may represent the "intermediate dentine" of other workers, and appears to be rich in polysaccharide and especially in acid mucopolysaccharide. The rest of the intertubular matrix, external to the first zone, showed practically no staining with methylene blue or toluidine blue at pH 2.6 or 3.6, and a reduction in the depth of PAS staining. It is considered that calcification of the intertubular dentine is virtually completed in the first zone.

The peritubular matrix first appeared about 60–100 μ from the predentine–dentine junction and quickly reached its full width. The peritubular matrix stained intensely and metachromatically with methylene blue and toluidine blue at pH 2.6 and 3.6 and deeply with alcian blue at 2.6. Observations made suggest that the peritubular matrix rapidly attains a high level of calcification.

The formation and calcification of the intertubular and peritubular matrices appear to be entirely separate stages in dentine development.

INTRODUCTION

THE NUMBER of histochemical studies of dentine that has been carried out is small. Those that have been concerned at least partly with human dentine are represented by the investigations of WISLOCKI, SINGER and WALDO (1948), WISLOCKI and SOGNAES (1950), BRADFORD (1951) and more recently WEILL (1959). Since the first two of these investigations were carried out before the existence of a radio-opaque, peritubular zone was demonstrated by BRADFORD (1950, 1951, 1955), MILLER (1954), SHROFF, WILLIAMSON and BERTAUD (1954), BERGMAN and ENGELDT (1955), RÖCKERT (1956), FRANK (1959) and TAKUMA (1960), the findings require to be largely re-interpreted.

In this work the distribution of polysaccharides in human dentine has been studied, with particular reference to the development of the peritubular zone. Attention has been paid to the possible existence of a region of "intermediate" dentine.

MATERIALS AND METHODS

The material consisted of sound premolar and molar teeth which had been extracted for orthodontic reasons. In none of these teeth was the formation of the roots completed: in some barely half the root area was formed. The material was fixed in 10% neutralized formol-saline. None of the material was decalcified. Serial sections were cut with 0.005 in. thick aluminium oxide (Aloxite) rubber bonded cutting wheels, supplied by the Carborundum Company, Trafford Park, Manchester, running at 6150 rev/min. During cutting the sections were cooled by a water spray. In the early stages of the work the sections were cut at 125 μ and subsequently reduced to a final thickness of 35–45 μ . In the later stages it was found possible to cut the sections serially at 80 μ . In both cases the reduction to 35–45 μ was obtained by an initial grinding between ground glass plates, the removal of the final 10 μ or so being accomplished by polishing the sections on a superfine Aloxite hone supplied by the Carborundum Company. At no stage were the sections allowed to dry.

In order to obtain sections cut at right angles to the course of the dentinal tubules for a proper study of the peritubular zones, each tooth was sectioned perpendicularly to its long axis through the coronal dentine between the pulpal cornua and the amelo-dentinal junction, as suggested by BRADFORD (1951). As the sections were cut serially, it was possible to follow the development of the peritubular zone from its first appearance near the predentine-dentine junction to its full formation as a broad, highly translucent zone farther out in the dentine.

The histochemical techniques used were staining with methylene blue, toluidine blue, alcian blue and the PAS method.

Methylene blue was used at a strength of 0.0005 M in either MCILVAINE'S (1921) phosphate-citric acid buffer or the acetate-barbiturate buffer of MICHAELIS (1931); in both instances at pH levels of 2.6, 3.6 and 7.0. The sections were stained for 24 hr. After staining, some sections were examined in water and subsequently blotted, then placed in cedarwood oil for an hour, passed into mixtures of cedarwood oil and benzene up to pure benzene and finally mounted in D.P.X. Other sections were carefully dehydrated in graded alcohols, passed through alcohol-benzene mixtures (two-thirds alcohol and one-third benzene; alcohol and benzene in equal parts; one-third alcohol and two-thirds benzene), placed briefly in benzene, and mounted in D.P.X.

Staining with toluidine blue was carried out with a 0.01% solution in the same buffer solutions as for methylene blue, for 12 hr: the subsequent treatment and mounting was identical. Some sections, however, were stained at pH 7.0 in 0.1% toluidine blue for 12 hr. These sections were stained when they were about 55–60 μ and subsequently brought to the usual thickness of 35–45 μ . This was done to eliminate any purely superficial staining.

For staining with alcian blue, the sections were placed for half an hour in freshly made and filtered solutions of the dye at strengths of 0.05–0.5% in the same range of buffer solutions as for methylene blue and toluidine blue. The sections were either dehydrated in alcohol, cleared in benzene and mounted in D.P.X., or were mounted in Aquamount (E. Gurr, Ltd.).

In the PAS technique, the Schiff reagent which was used was prepared by DE TOMASI's method (PEARSE, 1960, p. 822) as this was found to give better results than that produced according to the method of BARGER and DELAMATER (PEARSE, 1960, p. 822). The time of immersion of the sections in the periodic acid solution and the Schiff reagent was somewhat reduced from that of the usual procedure as it was found that, otherwise, the fully developed peritubular zones were disrupted. The optimal procedure was found to be 2 min in 1% periodic acid followed by 20 min in the Schiff reagent. As even the most careful dehydration with alcohol and the subsequent clearing also damaged the peritubular zones the sections were mounted in Aquamount.

RESULTS

Observations at low magnifications

On examination of sections in water after staining with methylene blue at pH levels of 2.6 and 3.6 it was possible to distinguish three regions or zones in the dentine proper; that is, excluding the predentine. Staining in either McIlvaine's or Michaelis' buffer gave very similar results, except that in Michaelis' buffer the staining was less intense and the colouring was generally of a more bluish shade. These zones were also found in those sections which were passed through cedarwood oil and benzene before mounting (Fig. 1). The three zones were distinguished by differences in the depth or colour of the staining (Table 1). No distinction was made at low power examination between the staining of intertubular and peritubular matrix.

The innermost zone was a narrow region of dentine adjacent to the predentine, and about 20–25 μ wide. This zone was discernable at all levels of pH employed.

The second zone was a rather broader one, ranging from 40 to 80 μ in width, and external to the first zone. It was readily seen at pH 2.6, at which level it was colourless, and so contrasted with the staining of the other two zones. At pH 3.6 a faint coloration appeared in it.

The rest of the dentine, which was external to the second layer or zone and formed the bulk of the tooth, constituted the third zone. This part of the dentine was stained with methylene blue at pH 2.6 and 3.6.

TABLE 1. METHYLENE BLUE STAINED SECTIONS (EXAMINED IN WATER)

	pH 2.6	pH 3.6
Predentine	Colourless	Colourless
Zone 1	Faint lavender	Deep lavender
Zone 2	Colourless	Faint lavender (narrowed)
Zone 3	Violet	Deep violet

At pH 7.0 a different pattern of staining was found (Fig. 2). Immediately adjacent to the predentine there was intense staining; elsewhere the dentine matrix was only

faintly stained. The dentinal tubules and their contents were, however, deeply stained in a zone surrounding the pulp cavity and extending for approximately 120–200 μ from the predentine–dentine junction; that is, this belt reached somewhat farther into the dentine than the limit of the second zone seen at the low pH levels. Beyond this the dentinal tubules were stained but much less deeply. At pH 7.0 the colouring was more blue compared with the lavender, or violet at the lower pH values.

When sections which had been stained with methylene blue at pH 2.6 or 3.6 were dehydrated in alcohol before clearing and mounting, certain differences in the pattern of the staining were found as compared with that described above. Both the innermost or first zone and the second zone were unstained. These differences were particularly marked when the sections were taken slowly through the alcohols. The staining of the third zone was altered less; there was a reduction of intensity at each level of pH.

Sections stained with toluidine blue at pH levels of 2.6, 3.6 and 7.0 and examined in water showed a similar pattern of staining to sections correspondingly treated with methylene blue; at pH 3.6, however, the second zone was less evident (Table 2). Sections stained with toluidine blue at pH 2.6 and 3.6, and dehydrated in alcohol before clearing and mounting, also showed a similar pattern of staining to the corresponding methylene blue stained sections.

TABLE 2. TOLUIDINE BLUE STAINED SECTIONS (EXAMINED IN WATER)

	pH 2.6	pH 3.6
Predentine	Virtually colourless	+
Zone 1	Blue	++
Zone 2	Pale blue-green	+
Zone 3	+	++

The sign + indicates the degree of metachromasia (reddish-purple).

With both methylene blue and toluidine blue the predentine was in general unstained at pH 2.6 and 3.6. The staining of the predentine was always less than that found in the innermost zone of the dentine (Tables 1 and 2).

With alcian blue, throughout the range of pH employed, there was a narrow region of deeply stained dentine immediately adjacent to the predentine–dentine junction. At pH 2.6 and 3.6 the staining in the rest of the dentine was confined to the course of the dentinal tubules, the degree of staining gradually diminishing as the amelo–dental junction was approached. At pH 2.6 especially, there was a somewhat less intense degree of staining in the region corresponding to the second zone of the methylene blue stained sections. The depth of staining everywhere in the dentine was most intense at pH 2.6. At pH 7.0 the pattern of staining was similar to that found in sections stained at pH 7.0 with methylene blue and toluidine blue. The predentine was most deeply stained at pH 7.0 and was only faintly coloured at pH 2.6.

With the PAS method the most intense staining was in a narrow region of dentine adjacent to the predentine. External to this the depth of staining gradually increased, except for the dentinal tubules and their contents which were intensely stained to a distance of about 120–200 μ from the dentine–predentine junction (Fig. 3). This was similar to the pattern of staining of the dentinal tubules found with methylene blue, toluidine blue and alcian blue at pH 7.0. The predentine was stained to a moderate intensity; considerably less than that of the adjacent dentine.

Observations at high magnifications

At high magnifications it was clear that the staining with methylene blue or toluidine blue at pH 2.6 and 3.6 of the innermost or first zone was almost entirely produced by the intertubular matrix. At pH 3.6 there was a faint staining of the periphery of the tubule. There was no evidence in this zone of any peritubular matrix.

In the second zone the faint staining at pH 3.6 was confined to the periphery of each tubule. There was no evidence in this region of peritubular matrix proper.

In the third zone, the intertubular matrix showed a similar reaction with methylene blue and toluidine blue at pH 2.6 and 3.6 to that of the second zone. The invariably greater depth of staining of the third zone was due entirely to the heavy staining of the peritubular matrix, the periphery of the tubules and to a lesser degree of the odontoblast processes (Tables 1 and 2).

The first sign of peritubular matrix was found in the innermost (i.e. most pulpal) part of the third zone (Fig. 4). It appeared there as a narrow but deeply stained area of matrix around each odontoblast process. This staining was found with both methylene blue and toluidine blue at pH 2.6 and 3.6, and in sections examined either in water or dehydrated with alcohol before clearing and mounting. The peritubular matrix very quickly assumed a greater thickness so that about 120–200 μ from the predentine–dentine junction, it had reached the maximum dimensions to be found in these sections (Fig. 5). Throughout the rest of the dentine the peritubular matrix appeared as a solidly stained zone, when examined in water.

In sections which had been passed through alcohol before mounting, however, a solidly stained peritubular zone was not found beyond the innermost part of the third zone (Fig. 6). Instead, the limits of each peritubular zone were marked by two deeply staining circles. These were more or less concentric; the outer at the junction of the intertubular and peritubular matrices and the inner at the periphery of the tubule and/or the outer limit of the odontoblast process.

At pH 7.0, the peritubular matrix was unstained except in the innermost part of the third zone where there was a narrow border of staining surrounding each tubule (Fig. 7). The unstained peritubular zone was easily distinguished from the intertubular matrix by its greater translucency (Fig. 8). The tubule or the odontoblast process was indicated by a circle of staining at the inner border of each translucent peritubular zone.

With alcian blue, the staining of the peritubular matrix was similar to that found with methylene blue and toluidine blue, in sections examined in water. This staining was most intense pulpally and at pH 2.6.

In sections treated by the PAS method, the odontoblast processes were densely stained to a distance of about 120–200 μ from the predentine–dentine junction. They did not, however, contrast strongly with the intertubular matrix surrounding them as it was also stained. Beyond this, the peritubular zone gradually became apparent as an unstained area surrounding the odontoblast process, which contrasted with the surrounding intertubular matrix even though the staining of this matrix was now greatly reduced (Fig. 9). Here, the outer limit of the odontoblast process, or the periphery of the tubule, stained quite strongly.

DISCUSSION

The histochemical methods employed in this investigation demonstrate the presence of polysaccharide and particularly of acid mucopolysaccharide. It is now generally accepted that staining of a tissue with methylene blue below pH 4.0 demonstrates the presence of acid mucopolysaccharide provided that nucleic acids can be excluded (PEARSE, 1960, p. 255). Further confirmation of the presence of acid mucopolysaccharide is provided by metachromasia with toluidine blue, especially at low pH, and staining with alcian blue at low pH (MOWRY, 1956). According to LISON (1953) true metachromasia is found with toluidine blue below pH 3.0. The PAS method indicates the presence of polysaccharide in general.

In the first, narrow zone in the dentine which was stained by methylene blue, the intertubular matrix was stained below pH 4.0 and at as low as pH 2.6. With toluidine blue it stained at pH 2.6 and 3.6 and showed metachromasia at pH 3.6. With alcian blue at all levels of pH employed, there was intense staining immediately adjacent to the predentine. These findings indicate a high content of acid mucopolysaccharide. The presence of some additional polysaccharide, however, is suggested by the intense PAS staining, for areas rich only in acid mucopolysaccharide are likely to show no reaction with the PAS method (PEARSE, 1960, p. 235).

LEBLOND, BÉLANGER and GREULICH (1955) described a strong metachromatic reaction at the predentine–dentine junction with toluidine blue; IRVING (1958) reported staining of this region with sudan black in sections which have previously been treated with hot pyridine; this was taken to be due to the presence of acid mucopolysaccharide. Moreover, an uptake of S^{35} was found at the predentine–dentine junction 2 hr after injection (BÉLANGER, 1954).

In the second zone, no staining with methylene blue or with toluidine blue was found in the intertubular matrix at pH 2.6 or 3.6. With the PAS method, apart from the odontoblast processes, there was a gradual diminution throughout this zone from the intense staining found at the predentine–dentine junction. Absence of acid mucopolysaccharide, and a gradual fall in the amount of other polysaccharide compared with the first zone appear to be indicated.

These findings suggest that the first zone in the dentine is the region where active changes associated with calcification are taking place. A high content of acid mucopolysaccharide is usually found in areas where calcification takes place (SOBEL, 1955). The greatly reduced staining in the second zone indicates a rapid fall from

the level of activity found in the first zone, so that at a distance of about 20–25 μ from the predentine–dentine junction the intertubular matrix must be already well advanced towards its final state.

In the third zone, the virtual absence of staining of the intertubular matrix with methylene blue and toluidine blue at pH 2.6 and 3.6, and still further reduced PAS reaction, appear to confirm that little change is taking place in this matrix.

The first zone would appear to correspond to a certain extent with the “intermediate dentine” which has been described by SCHOUR (1960). SCHOUR, however, described the intermediate dentine as the border farthest from the pulp where the predentine becomes calcified, and as representing an early stage of the calcification process.

The staining of the third zone, which constitutes the bulk of the dentine of each tooth, with methylene blue at pH 2.6 and 3.6 is due almost entirely to the peritubular matrix. The peritubular matrix first appears about 60–100 μ from the predentine–dentine junction, that is, a little way into the calcified dentine. This confirms the findings of BLAKE (1958) in that respect.

The intense staining of the peritubular matrix in the third zone with methylene blue at pH 2.6 and 3.6, the metachromasia with toluidine blue at these levels, and the deep staining with alcian blue at pH 2.6, all indicate a rich content of acid mucopolysaccharide. It seems likely, however, that rapid formation of the peritubular matrix and its calcification to a high level take place in the innermost part of the third zone. This view is supported by the observation that, farther out in the dentine, the peritubular zone shows an absence of staining with methylene blue and toluidine blue at these pH levels in sections which had been dehydrated in alcohol; apart, that is, from the two concentric circles already described. This is consistent with a highly calcified peritubular zone which would be decalcified to a considerable extent by immersion in solutions at low pH for 12–24 hr and then disrupted by dehydration with alcohol. Where the time in acid solution is short as with the PAS method or with alcian blue, and dehydration with alcohol is avoided, the peritubular zone remains intact. With the PAS method, the peritubular zone in this region is translucent and unstained; this suggests at the most a very low level of polysaccharide other than acid mucopolysaccharide.

The results obtained with methylene blue and toluidine blue at pH 7.0 are in accord with the concept of a peritubular matrix which, throughout its course, is so calcified that it does not stain, except in the innermost formative region. Here, a reactive surface exists which is capable of taking up stain.

It is noteworthy that the staining of the peritubular matrix with methylene blue and toluidine blue at pH 2.6 and 3.6 remains after passage through alcohol unlike the staining of the first zone. From this it may be inferred that the acid mucopolysaccharide content of the peritubular matrix must differ in type or state from that of the first zone. The staining of the peritubular matrix with methylene blue at pH 2.6 and 3.6 moreover, is different in colour from that of the intertubular matrix of the first zone (Table 1).

It seems clear from these results that WISLOCKI *et al.* (1948) only examined areas of dentine where the peritubular zones were already well formed and highly calcified. With toluidine blue it was observed that "the dentinal tubules are stained a pronounced lavender or purple. The color is sharply localized in the wall of the tubules immediately bordering the lumen. This inner zone of the dentinal tubules, designated as Neumann's sheath . . .", and "The lumen of each tubule is occupied by an unstained dentinal fiber (Tomes' fiber) . . .". With the PAS method it was found that ". . . Neuman's sheaths are unstained". Since the existence of a highly calcified translucent peritubular zone had not then been demonstrated, the unstained zone with the PAS method was taken as corresponding to the narrow belt of staining with toluidine blue which was found at the periphery of the dentinal tubule, the so-called Neumann's sheath. The diagrams used in illustration of their paper show the different dimensions of the two features.

In both the first and second zones, a thin line of staining appeared at the periphery of each dentinal tubule with methylene blue and toluidine blue at pH 3.6. It is possible that a continuation of this structure forms the inner circle of staining related to the peritubular matrix, this was thought to represent either the periphery of the tubule or the outer limit of the odontoblast process. It may be that this structure corresponds to the immature juxta-cytoplasmic zone, described by WEILL (1959), which runs along the length of the dentinal tubules and their branchings.

Little attention has been given to the predentine in this investigation. It has been noted, however, that the predentine was unstained with methylene blue at pH 2.6 and 3.6, showed some metachromasia with toluidine blue at pH 3.6, but not at pH 2.6, and was only faintly stained with alcian blue at pH 2.6. There would, therefore, appear to be a relative absence of acid mucopolysaccharide in the predentine. The fact that the predentine showed deep staining with alcian blue at pH 7.0 supports the contention of MOWRY (1956) that alcian blue gives more reliable results at a very low pH. WEILL (1959) has described two zones in the predentine, the first adjacent to the odontoblasts and coloured deeply by toluidine blue at pH 2.9, the second adjacent to the dentine and in which the matrix remained colourless. It may be that the predentine observed in the present work corresponds to the second zone of WEILL. After the final preparation of the sections it was uncommon to find any trace of the odontoblast layer still present; thus a delicate juxta-cytoplasmic zone of predentine might well have been lost.

Acknowledgement—I am greatly indebted to Mr. D. A. FINLAYSON and Mr. H. R. HEALD for supplying the teeth used in this investigation.

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PLATE I

FIG. 1. Section through the pulp cavity and adjacent dentine; stained with methylene blue at pH 2.6. No predentine is to be seen. The pulp tissue was lost in preparation of the section. Mounted in D.P.X. after passage through cedarwood oil and benzene. $\times 54$.

FIG. 2. Section through the pulp cavity and adjacent dentine; stained with methylene blue at pH 7.0. No predentine is to be seen. The pulp tissue was lost in preparation of the section. Mounted in D.P.X. after passage through cedarwood oil and benzene. $\times 54$.

FIG. 3. Section through the pulp cavity and adjacent dentine; stained with the PAS method. No predentine is to be seen. The pulp tissue was lost in preparation of the section. Mounted in Aquamount. $\times 54$.

FIG. 4. Transverse section through the dentinal tubules at the pulpward margin of the third zone. Here there is the first appearance of the peritubular matrix. Stained with methylene blue at pH 3.6, dehydrated in alcohol, and mounted in D.P.X. $\times 1200$.

FIG. 5. Transverse section through the dentinal tubules at a level where the full thickness of the peritubular matrix has appeared. Two tubules may be seen in relation to which no peritubular matrix has formed. Stained with methylene blue at pH 3.6, dehydrated in alcohol and mounted in D.P.X. $\times 1200$.

FIG. 6. Transverse section through the dentinal tubules at a level where fully developed, translucent, peritubular zones have appeared. The area is farther away from the pulp than that shown in Fig. 5. Stained with methylene blue at pH 3.6, dehydrated in alcohol, and mounted in D.P.X. $\times 1200$.

FIG. 7. Transverse section through the dentinal tubules at a level where formation of the peritubular matrix is taking place. Stained with 0.1% toluidine blue at pH 7.0. Mounted in D.P.X. after passage through cedarwood oil and benzene. After staining the section was polished on a superfine Aloxite hone to remove any purely superficial staining. $\times 1200$.

FIG. 8. Transverse section through the dentinal tubules at a level where fully developed, translucent, peritubular zones have appeared. The area is similar to that shown in Fig. 6. Stained with 0.1% toluidine blue at pH 7.0. Mounted in D.P.X. after passage through cedarwood oil and benzene. After staining the section was polished on a superfine Aloxite hone to remove any purely superficial staining. $\times 1200$.

FIG. 9. Transverse section through the dentinal tubules where fully developed, translucent, peritubular zones have appeared. The area is similar to those shown in Figs. 6 and 8. One tubule at the top right hand corner of the field shows an absence of a translucent peritubular zone. PAS method. $\times 1200$.

HISTOCHEMICAL STUDY OF DENTINAL INTER- AND PERITUBULAR MATRICES

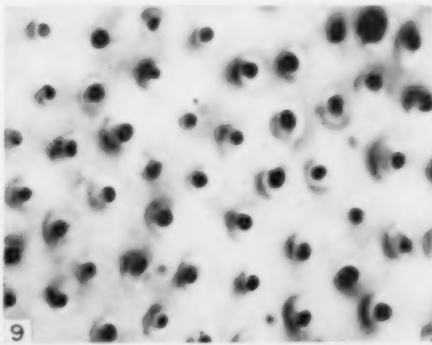
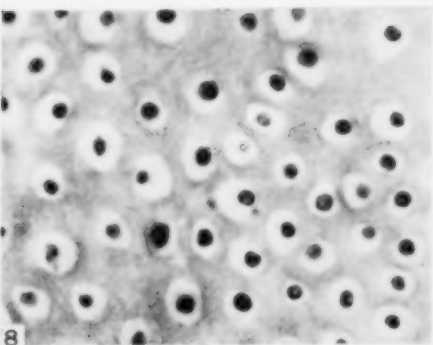
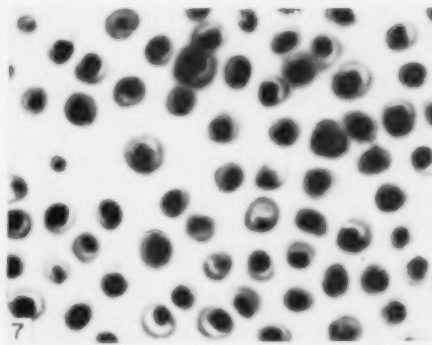
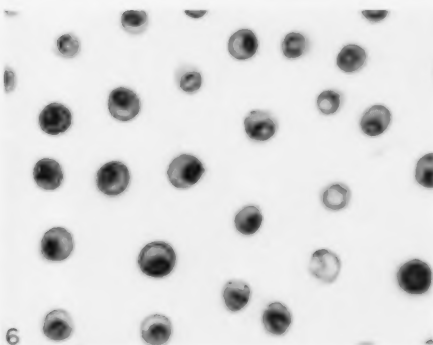
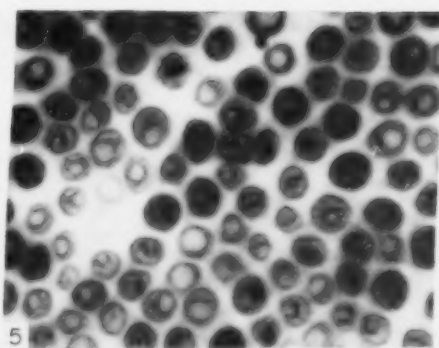
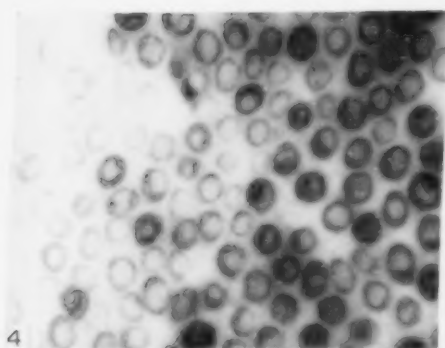
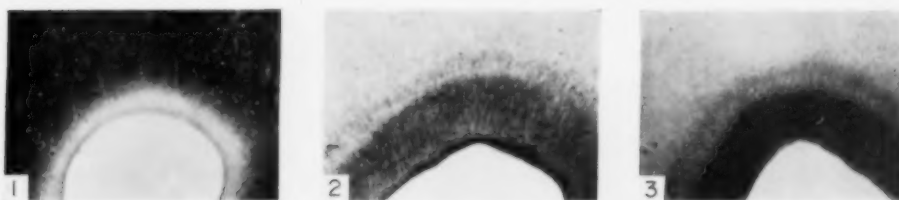


PLATE I

Vol.

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MOLECULAR SIEVE BEHAVIOUR OF NORMAL AND CARIOUS HUMAN DENTAL ENAMEL

A. I. DARLING, K. V. MORTIMER, D. F. G. POOLE and W. D. OLLIS*

Dental School and *Department of Organic Chemistry,
University of Bristol, England

Abstract—In an attempt to understand the basis for the histological appearances of the dark zone of enamel caries a detailed study of it was made by polarized light in various media. It is shown to contain a system of minute spaces constituting a molecular sieve. As a result of this system, liquids with small molecules, such as water and methanol, may penetrate the dark zone freely, whereas the degree of penetration of liquids with larger molecular depends upon the size of the molecule. Such features are shown to varying degrees in both normal enamel and the translucent zone of the carious lesion. The spaces in the body of the lesion are sufficiently large to allow the inclusion of almost all liquids.

Extensions of such studies on the three zones of the carious lesion and normal enamel allowed the percentage volume and approximate size of the spaces present in these areas to be assessed. On this basis the histological appearances of normal and carious enamel as seen in ground sections viewed by ordinary and polarized light in various media may be explained.

INTRODUCTION

IN A PREVIOUS paper (DARLING, 1956) the behaviour of the dark zone around the margin of the carious lesion of the enamel was described. In this and a subsequent paper (DARLING, 1958), the behaviour of the enamel in polarized light was discussed, and the basis for its birefringence in various media was explained. When a tissue such as enamel containing spaces is imbibed with a medium of refractive index different from that of the tissue two main factors are responsible for the birefringence as observed in polarized light. The first of these is the intrinsic birefringence of the tissue which in the case of the enamel is negative in relation to the prisms. This is a direct effect on the polarized light due to the regular arrangement of the crystallites of apatite within the enamel. It is possible that the intrinsic birefringence is modified to some degree by the organic matrix, though this modification is probably very small. The second factor is form birefringence which is the result of a medium of refractive index different from that of the enamel occupying spaces within the enamel which are small as compared with the wavelength of light. This form birefringence is positive in relation to the prisms and the amount produced depends on the relative volume of spaces present and on the difference between the refractive index of the enamel and that of the medium within the spaces. In any specimen, as the difference between these refractive indices increases so the amount of form birefringence also increases. In these circumstances the tissue behaves as a Wiener mixed body and the observed birefringence is a summation of the intrinsic birefringence and the form

birefringence. When the observed birefringence in various media is plotted against the refractive index of the medium, it gives a smooth curve which is known as the imbibition curve. In the measurement of intrinsic birefringence it is necessary to eliminate form birefringence so the spaces in the specimen must be filled with a medium of the same refractive index as enamel (1.62). The technique used for the determination of birefringence was that described by DARLING (1956). Throughout the paper the figures given for birefringence are in fact birefringence $\times 10^4$ to avoid the use of several places of decimals.

As previously described (DARLING, 1956, 1958) the enamel of the carious lesion in both the dark zone and the body of the lesion behaves as a Wiener mixed body so long as it is observed in aqueous media. Previous observations (DARLING, 1956) show that while the body of the lesion continues to behave in this manner in non-aqueous media the dark zone does not show this behaviour in most non-aqueous media. The dark zone shows an excessive amount of form birefringence which appears to vary considerably with the medium used regardless of its refractive index. In the same paper (DARLING, 1956) it was stated that the most probable basis for the production of this excessive form birefringence in the dark zone by non-aqueous media and its absence in aqueous media was the action of the imbibition medium on the organic matrix. In transferring the specimens from aqueous media to non-aqueous media it was necessary to dehydrate them with absolute ethanol and it was always noticed that the unusual form birefringence developed first in transfer from water to absolute ethanol. The change was reversible and at that time it seemed most probable that it was caused by dehydration of the organic matrix. However, in the same paper it was noted that in methanol the dark zone behaved in precisely the same manner as in water (Fig. 1). This point was not discussed at that time but it is now clear that this

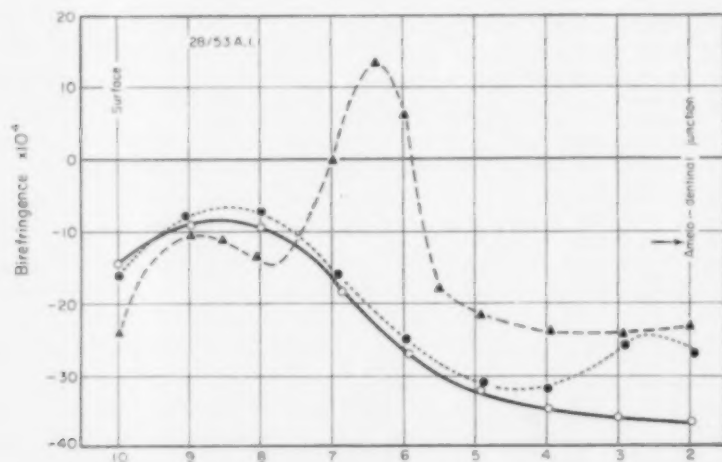


FIG. 1. Observed birefringence of a series of points through a longitudinal ground section of a carious lesion in various media. Ethanol (\blacktriangle), methanol (\bullet), water (\circ).

was a crucial observation because as methanol is itself a very potent dehydrating agent and does not produce the unusual form birefringence in the dark zone, this phenomenon cannot be caused solely by dehydration. It was therefore necessary to re-examine changes seen in the dark zone in various media in an attempt to understand precisely how they are produced and what they can reveal of the structure of the carious lesion at this point. The studies now reported show that the original interpretation was not correct and a new explanation is given.

The present work falls conveniently into two parts starting with the preliminary investigations on which a new explanation was based and continuing into a re-examination of the various zones of the carious lesion and normal enamel to see how the explanation fits the findings in these areas.

PRELIMINARY INVESTIGATIONS

Material and methods

In the course of these investigations five specimens from five different teeth have been used. All were ground sections, approximately plano-parallel and from 50 to 125 μ in thickness. When not in use they were stored in 70% ethanol. These sections showed typical early carious lesions which had not involved more than half the depth of the enamel and in which no cavitation had occurred. Four lesions were situated approximately and one was related to an occlusal fissure.

(1) A single point in the dark zone of each of two carious lesions was examined in polarized light and the observed birefringence was recorded after imbibition first with absolute ethanol and then with mixtures of ethanol and water progressing to pure water. This procedure was then repeated with the media in the reverse order from water to ethanol. The specimen was left in each medium for approximately 30 min.

(2) Similar points in the dark zones of each of two carious lesions in two of the sections were then examined in precisely the same way in mixtures of methanol and water starting from absolute methanol and progressing through various dilutions to pure water and then proceeding in the reverse direction.

(3) Similar points in the same carious lesions as those examined in (1) were examined in mixtures of ethanol and methanol. Because the amount of unusual form birefringence was most marked in absolute ethanol, it was decided to commence with absolute ethanol and progress to absolute methanol before proceeding in the reverse order from absolute methanol to absolute ethanol. Readings were taken in the same way as before.

(4) Similar points in the same carious lesions as those examined in (1) and (3) were again examined in mixtures of quinoline and ethanol commencing with absolute ethanol, progressing to pure quinoline and then proceeding in the reverse direction back to absolute ethanol. The readings were taken in the same manner as previously described. Because quinoline did not develop its typical effects within half an hour the readings in pure quinoline and in absolute ethanol were taken after imbibition overnight.

Results of preliminary investigations

Typical results from the four different procedures are shown in Figs. 2-5. In preliminary investigation (4) (Fig. 5) because of the variation in the times of exposure to media of different concentrations, as stated above, these results do not give a true picture of the maximum effects in the various media, but the two such points in pure quinoline and absolute ethanol are probably quite accurate. The intermediate points are probably modified by insufficient exposure to the media.

Discussion of preliminary investigations

For the purpose of this paper the terms "rise" or "fall" in birefringence should be interpreted as meaning that the birefringence becomes more positive or more negative respectively.

The term "passage" when used in a context such as "passage from ethanol to water" means that the specimen was examined first in absolute ethanol and then in various mixtures of water and ethanol in which the concentration of water was progressively increased until the final examination was made in pure water.

The term "transfer" is used only when the specimen is examined first in one medium and then in another without the intervention of other media.

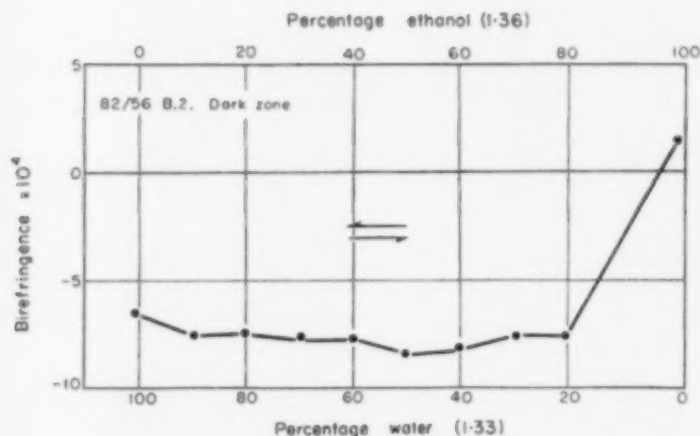


FIG. 2. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with various mixtures of water and ethanol. Slight variations occurred in the readings from water to ethanol, and in the reverse direction but no significant difference was detected.

The readings in mixtures of ethanol and water (Fig. 2) show a slight fall in observed birefringence from pure water to 50% ethanol after which there is a slight rise in observed birefringence to 80% ethanol and a sudden and considerable rise in observed birefringence from 80% to 100% ethanol. The fall from 0% to 50% ethanol is consistent with the slight difference in refractive index between water (1.33) and ethanol

(1.36). If the specimen were behaving as a Wiener mixed body one would expect a slight fall between water and ethanol due to a change in form birefringence. The sudden rise in observed birefringence between 80% and absolute ethanol is consistent with previous findings (DARLING, 1956). This reversible change must be due to form birefringence but whatever the explanation it cannot be caused by the difference in refractive index between 80% and absolute ethanol.

The results from observations of the dark zone in mixtures of methanol and water (Fig. 3) show a slight rise in observed birefringence from pure water to absolute methanol. The pattern is quite different as there is no sudden rise when absolute methanol is approached. The slight rise in the observed birefringence from water to methanol is consistent with the slight difference in refractive index which might be expected to produce this change between these two media (water 1.33, methanol 1.329). The absence of any gross rise in observed birefringence as absolute methanol is approached proves conclusively that the effect seen in ethanol is not solely due to dehydration, as methanol is itself a potent dehydrating agent.

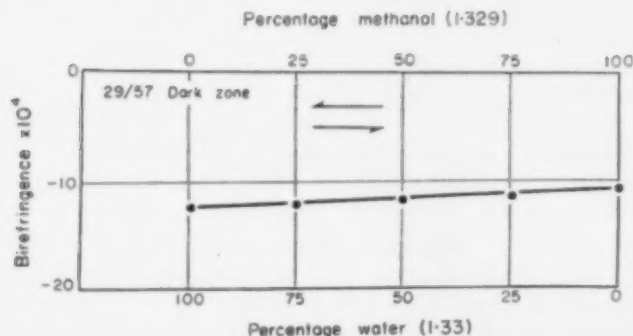


FIG. 3. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with various mixtures of water and methanol. The readings were almost identical in both directions.

The observed birefringence of the dark zone during passage from ethanol to methanol and back again (Fig. 4) shows that the effects produced are not reversible. In passage from methanol to ethanol (dotted line) the results are comparable with those obtained by passage between methanol and water (Fig. 3) and are susceptible to the same explanation that they are caused by changes in form birefringence which are consistent with the differences in refractive index between the media (methanol 1.33, ethanol 1.36). It is worth noting that this is true although both methanol and ethanol are dehydrating agents. Clearly the results from passage in either direction cannot be due to dehydration. In passage from ethanol to methanol the results (Fig. 4 continuous line) show a fall in observed birefringence between absolute and 50% ethanol in methanol. This is comparable with, though less dramatic than, the effect of passage from ethanol to water and must be explained on the same basis. It

must be caused by a high form birefringence in absolute ethanol which is unrelated to the refractive index of this medium and which can be abolished by both methanol and water.

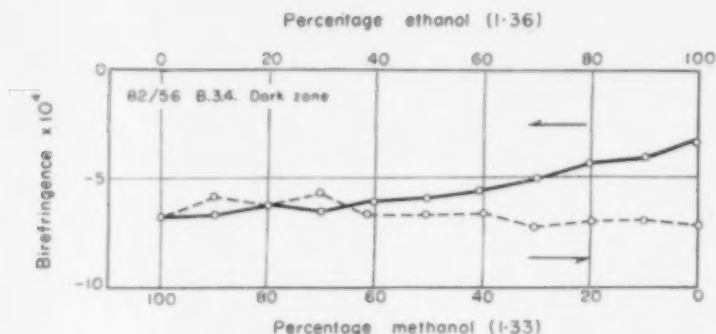


FIG. 4. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with various mixtures of methanol and ethanol. The readings in both directions are given.

In passage from absolute ethanol to quinoline (Fig. 5) there is an even greater rise in observed birefringence than that found in passage from water to ethanol (Fig. 2). Here it must be presumed that the section as examined in absolute ethanol was already well dehydrated and it seems unlikely that further dehydration occurred in its passage to quinoline. Thus it seems unlikely that the rise in observed birefringence found in passage from ethanol to quinoline is a result of dehydration. As in the case of passage between water and ethanol, the change seen in passage from ethanol to quinoline is reversible and must be due to form birefringence, but the amount of form birefringence produced cannot be caused by the refractive index of the medium, as quinoline (1.62) has the same refractive index as enamel.

DARLING (1956) showed that the dark zone behaved as a Wiener mixed body in aqueous media. It is now clear that this zone probably behaves in the same manner in methanol (Fig. 3). However, in ethanol and quinoline this is not so and unusual amounts of form birefringence are produced in both these media. It seems that the unusual form birefringence produced in quinoline is even greater than that produced in ethanol in spite of the fact that the refractive index of quinoline (1.62) is identical with that of enamel, while that of ethanol (1.36) is considerably different from that of enamel.

The possibility that these effects and those described later might be caused by chemical or physical interaction between the molecules of the media was considered but had to be rejected.

It was realized that another explanation was possible (DARLING and MORTIMER, 1960) and a preliminary report has been made (POOLE *et al.*, 1961). The spaces in the dark zone which produce the excessive form birefringence in non-aqueous media might be a constant feature of the carious lesion and not a manifestation of physical changes

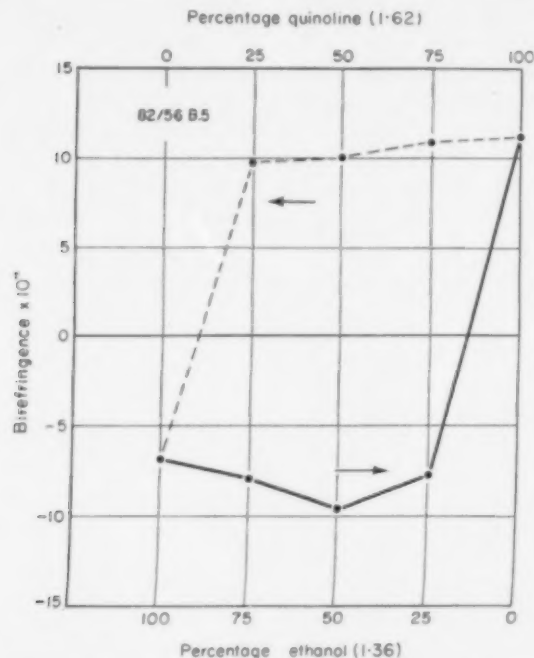


FIG. 5. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with various mixtures of ethanol and quinoline. The readings in both directions are given.

in the organic matrix as was presumed when the hypothesis of dehydration was advanced. This new proposal requires the assumption that the aqueous media and methanol can enter these spaces relatively freely while the other non-aqueous media cannot do so. The ability of a medium to enter the spaces might well depend on its molecular size as both water and methanol have small molecules, while that of ethanol is slightly larger, and that of quinoline is much larger.

On this basis it could be argued that water and methanol enter all the spaces so that the changes in form birefringence between these two media are solely dependent upon their refractive indices. This is in agreement with the findings in these media (Fig. 3). In passage from water to ethanol and back again (Fig. 2), the water would be able to enter all the spaces but the ethanol would not. In higher concentrations of ethanol the affinity between ethanol and water might cause some of the water molecules to be withdrawn from the spaces leaving them empty or filled with water vapour. This vapour would have a very low refractive index thus giving a very high form birefringence. In passage from ethanol to methanol and back again (Fig. 4) the methanol would be able to enter the spaces but the ethanol would not. Thus the high form birefringence in ethanol would be eliminated as the methanol entered the

spaces. In passage from methanol to ethanol, however, it would appear that the methanol molecules remain in the spaces which are inaccessible to ethanol. Thus even in absolute ethanol after passage from methanol the unusual and excessive form birefringence is not produced.

The results of passage from quinoline to ethanol and in the reverse direction (Fig. 5) can be explained similarly since the molecular size of quinoline is very much greater than that of ethanol but it must be presumed that there is an affinity between quinoline and ethanol so that in passage from ethanol to quinoline the molecules of ethanol are drawn out of some spaces but are not replaced by the much larger quinoline molecules. In this way the rise in form birefringence in quinoline could be explained.

MAIN INVESTIGATION

The hypothesis described above seemed to offer such a reasonable explanation that it was decided to test it by a further series of investigations in which a variety of media with similar refractive indices but different molecular sizes was used. It was also decided to extend these investigations to cover all of the three zones of the carious lesion and normal enamel in addition.

Material and methods

The series of straight chain alcohols from methanol to *n*-octanol provided a convenient group of media with similar refractive indices but different molecular sizes (Table 1). All of the refractive indices fall within a range of 1.33–1.43 while five of them fall within a range of 1.40–1.43. Such differences would have little effect on form birefringence.

The molecular environment of the hydroxyl groups in these alcohols is so similar that it is unlikely to be directly concerned with the extent of their inclusion.

The shape of the molecules in this series is such that they all have very similar cross sections but vary considerably in length and therefore in volume. It was therefore decided to add to this series several branched chain alcohols. These included a variety of molecular shapes so that an effect due to variation in cross sectional area of the molecules might be detectable. The refractive indices of the branched chain alcohols all fell within the same range as that for straight chain alcohols (Table 1).

The specimens used were those previously described. Each specimen was dried in warm air after prolonged immersion in ethanol, it was then examined in air and transferred through the series of straight chain alcohols in descending order of molecular size to methanol, after which it was dried and passed through the branched chain alcohols when these were used. It was then washed and dried again to eliminate previous media and passed through water and the aqueous media (Thoulet's solutions, DARLING, 1956). After this it was again dehydrated in ethanol, dried in warm air and transferred to quinoline. In some cases, only a selection of the alcohols was used to reduce the time needed for each investigation. The branched chain alcohols were only used in the investigation of the dark zone. Each specimen was left in each medium for 24–48 hr except when the translucent zone was being studied. In this zone, it was

TABLE 1. THE OBSERVED BIREFRINGENCE OF A SINGLE POINT IN THE DARK ZONE OF A CARIOUS LESION OF THE ENAMEL WHEN IMBIBED WITH EACH MEDIUM IN TURN AS DESCRIBED IN THE TEXT

In each group of media the molecular size decreases as the group is descended

Medium	Formula	Refractive index	Observed birefringence $\times 10_4$
Straight chain alcohols			
<i>n</i> -Octanol	$\text{CH}_3(\text{CH}_2)_7\text{OH}$	1.43	+5.85
<i>n</i> -Heptanol	$\text{CH}_3(\text{CH}_2)_6\text{OH}$	1.42	+4.11
<i>n</i> -Hexanol	$\text{CH}_3(\text{CH}_2)_5\text{OH}$	1.42	+3.23
<i>n</i> -Pentanol	$\text{CH}_3(\text{CH}_2)_4\text{OH}$	1.41	-4.84
<i>n</i> -Butanol	$\text{CH}_3(\text{CH}_2)_3\text{OH}$	1.40	-5.85
<i>n</i> -Propanol	$\text{CH}_3(\text{CH}_2)_2\text{OH}$	1.38	-7.33
Ethanol	$\text{CH}_3\text{CH}_2\text{OH}$	1.36	-10.99
Methanol	CH_3OH	1.33	-11.97
Branched chain alcohols			
<i>tert</i> -Amyl	$\text{CH}_3\text{CH}_2\text{C}(\text{CH}_3)_2\text{OH}$	1.41	+12.59
<i>sec</i> -Butyl	$\text{CH}_3\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$	1.39	+11.97
<i>tert</i> -Butyl	$(\text{CH}_3)_3\text{C}(\text{OH})$	1.38	+11.39
<i>iso</i> -Propyl	$(\text{CH}_3)_2\text{CH}(\text{OH})$	1.37	+3.89
Allyl*	$\text{CH}_2=\text{CH}-\text{CH}_2\text{OH}$	1.41	-9.71
Other media			
Quinoline		1.62	+13.21
Water		1.33	-13.01
Air		1.00	+19.1

* Allyl alcohol is not a branched chain alcohol but is included in this group because of its molecular shape.

found that imbibition occurred much more rapidly. After the appropriate time for imbibition in each medium, observations were made with the aid of a polarizing microscope and a Berek compensator from which the observed form birefringence could be calculated. Two points were examined in the dark zones of two lesions, two points in the body of two lesions, four points in the translucent zones of three lesions and two points in normal enamel.

Results

Examples of the results are given in Table 1 and Figs. 7-10. The duplicate results were in each case similar to those shown. The branched alcohols have been omitted from Fig. 8 to avoid congestion but, as can be seen from Fig. 6, they behaved in a manner similar to that of the straight chain alcohols. In Figs. 7-10 it is fortunate that the size of molecule in the series of straight chain alcohols decreases with the

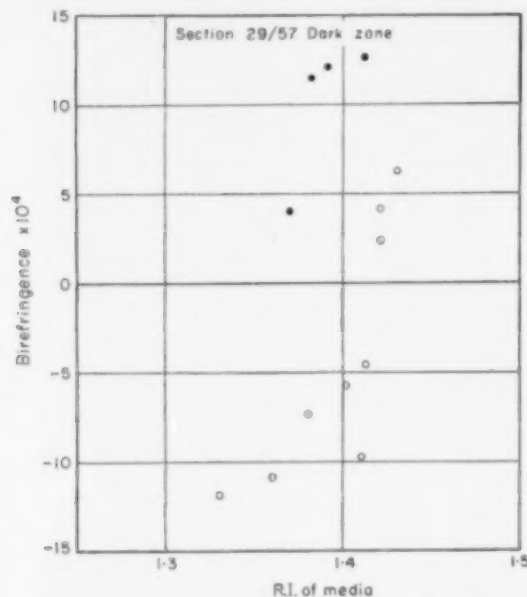


FIG. 6. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with straight chain and branched chain alcohols, plotted against the refractive index of the media. Straight chain alcohols (\circ), branched chain alcohols (\bullet).

refractive index so that *n*-octanol always appears on the right of the group and methanol on the left. Quinoline with a refractive index of 1.62 is always isolated on the extreme right of the graph. Other refractive indices are given in Table 1.

Discussion

From the results with straight chain alcohols (Table 1) it is quite clear that in the dark zone the amount of form birefringence depends primarily on the length of the molecule while from the results with the branched chain alcohols it is equally clear that the form birefringence depends also on the cross sectional area of the molecule. The assessment of molecular dimensions is extremely difficult and for this reason no attempt has been made to relate the two series of alcohols to each other. However, a comparison of *n*-propanol and *iso*-propanol is of interest. Examination of models of these molecules (Fig. 12) shows that although the dimensions are similar, their molecular shapes are very different. *Iso*-propanol has a much more awkward shape than *n*-propanol and is therefore more difficult to accommodate in the spaces. This is reflected in the amount of form birefringence produced which is greater with *iso*-propanol. As a result, the observed birefringence in this medium is more positive (Table 1). Fig. 6 shows the form birefringence produced, charted against the refractive indices of the alcohols. As can be seen there is no relationship between the refractive indices of the media and the form birefringence produced. Indeed if the changes in

refractive index of the alcohols had been influencing the observed birefringence then this would have produced a trend opposite to that observed in Table 1.

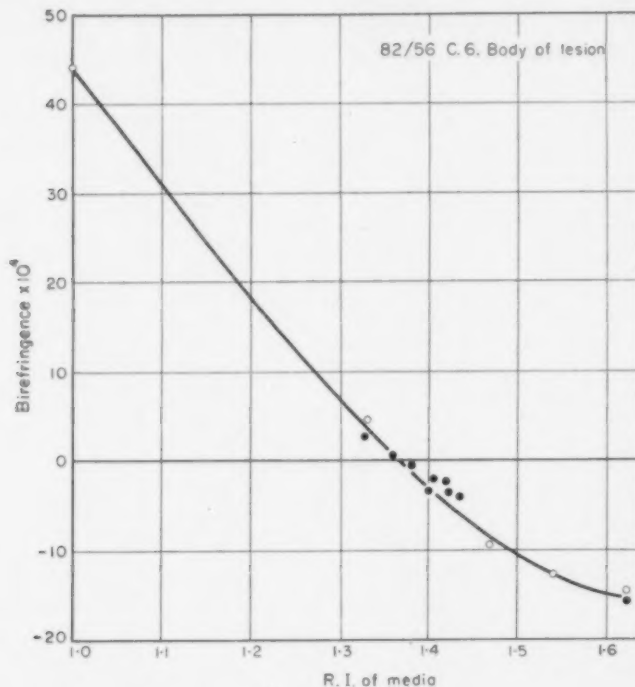


FIG. 7. Observed birefringence at a single point in the body of a carious lesion imbibed with some of the media listed in Table 1 plotted against the refractive index of the media. Watery media and air (O), alcohols and quinoline (●).

The fact that there is a relationship between the form birefringence produced and the shape and dimensions of the molecules of the medium implies that the dark zone is acting as a molecular sieve (BARRER, 1949). It must contain a series of spaces of various sizes. At one end of the series the spaces are accessible only to small molecules such as those of air, water and methanol. The spaces of larger size are accessible to the alcohols with larger molecules. Thus *n*-heptanol can enter more spaces than *n*-octanol, *n*-hexanol more than *n*-heptanol and so on down the series. Having started from air with all the spaces presumably filled by this medium each alcohol enters those spaces which are large enough to accept its molecules and the remaining spaces continue to be filled with air. The refractive index of air (1.0) is so different from that of enamel (1.62) that it produces a high degree of form birefringence even when few spaces are present.

From the results shown in Figs. 7-10 it can be seen that in each zone the readings with the aqueous media and air, fall on the same imbibition curve and in all except the

translucent zone the reading for methanol falls on the same curve. Thus it is clear that in these media the various zones are behaving as Wiener mixed bodies and all spaces are equally accessible to these media. As previously described by DARLING (1958) the form birefringence produced in the various media can be used to assess the percentage of imbibable spaces which are present. Form birefringence is calculated

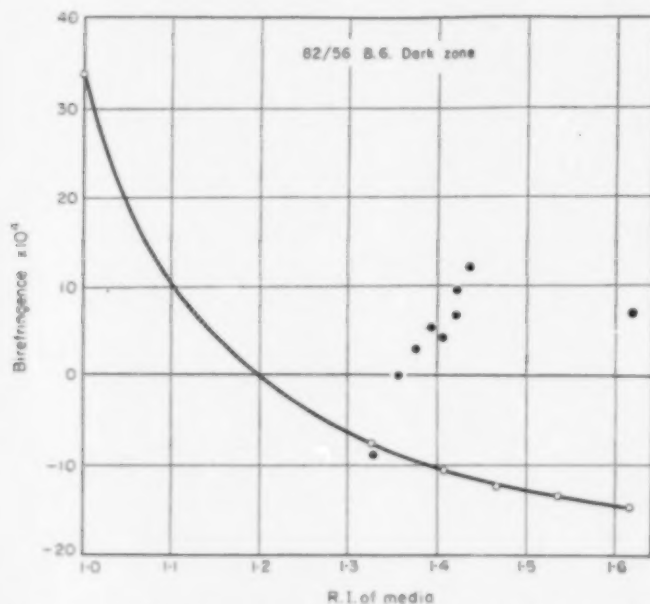


FIG. 8. Observed birefringence at a single point in the dark zone of a carious lesion imbibed with some of the media listed in Table 1 plotted against the refractive index of the media. Watery media and air (O), alcohols and quinoline (●).

by subtracting the intrinsic birefringence from the observed birefringence. Therefore this assessment depends on the accuracy of the readings in the various media and on the correct measurement of the thickness of the section. The value for the intrinsic birefringence $\times 10^4$ of normal enamel is usually in the range of 30–40 (DARLING, 1956). In comparison with this value the readings for intrinsic birefringence in the dark zone, the body of the lesion and normal enamel in one section (Figs. 7–9) were consistently low. As this probably arose from an inaccurate measurement of thickness it was decided to correct these readings to give an intrinsic birefringence of approximately 30. All readings from this section were corrected by the same factor. From the corrected readings it was then possible to assess the amount of form birefringence produced in each medium.

DARLING (1958) figured a graph (Fig. 11) of the amount of form birefringence produced in air and aqueous media by various percentages of spaces. This was based on a formula modified from that given by WIENER (1912). By using this graph and

reading off the corrected amount of form birefringence in each medium it was possible to assess the percentage of spaces present in the thickness of the section, in the various zones at the points examined. It soon became apparent that in aqueous media with refractive indices below 1.4 the variations due to experimental error played too great a part and these readings were therefore disregarded. Table 2 gives the percentage of spaces as assessed in the various zones.

TABLE 2. PERCENTAGE VOLUME OF SPACES AS ASSESSED FROM THE FORM BIREFRINGENCE IN AIR, IN WATER, AND FROM THE DIFFERENCE IN FORM BIREFRINGENCE BETWEEN AIR AND WATER

	Air (%)	Water (%)	Air-Water (%)
Normal enamel	0.1	0.1	0.1
Translucent zone	0.4	0.3	0.4
Dark zone	2.6	2.4	2.6
Body of lesion	—	6.4	—

Having demonstrated the existence of a molecular sieve in the dark zone it seemed at least possible that some spaces in the various zones were not penetrable by the complex salt of mercuric iodide and potassium iodide existing in Thoulet's solutions and that this might affect the assessment of the intrinsic birefringence. Ideally we should like to have examined the various points in a medium with a molecule small enough to penetrate all spaces and with a refractive index of 1.62 similar to that of enamel, but as such a medium was not available it was necessary to use other methods to check up on this point. What was required was a method of assessing the amount of spaces present, independently of the molecular size of the medium. The nearest approach to this was obtained by using the difference between the form birefringence produced in air and water. As both these media enter all the spaces equally, the difference in form birefringence would not be affected by molecular size, nor would it be dependent on the intrinsic birefringence. A graph, based on the previous one (Fig. 11), was drawn of the difference in form birefringence in air and water which should be produced with various percentages of spaces. From this it was possible to read off the amount of spaces necessary to produce the difference in form birefringence between air and water as measured in the specimens. Table 2 gives the total percentage of spaces assessed by this method and the percentage of spaces assessed directly from the form birefringence in water and air. It can be seen that there is very little difference between the various assessments. It should be mentioned here that readings in air were difficult in the body of the lesion because of the relative opacity of this zone when completely dried in air. For this reason, in this zone no attempt has been made to assess the spaces from the difference between the form birefringence in water and that in air. As can be seen from Table 2, the total amount of spaces in the thickness of the section, in the various zones examined, falls within the range as given by DARLING (1958) who stated that in the translucent zone there

was approximately 1 per cent of spaces, in the dark zone approximately 2-4 per cent of spaces and in the body of the lesion more than 4 per cent of spaces. As previously stated, the point chosen in the translucent zone (Fig. 9) was fairly close to the normal enamel, to reduce the effect of the dark zone upon it, while the point in the body of the lesion was fairly close to the dark zone because the lesion itself was quite small in size.

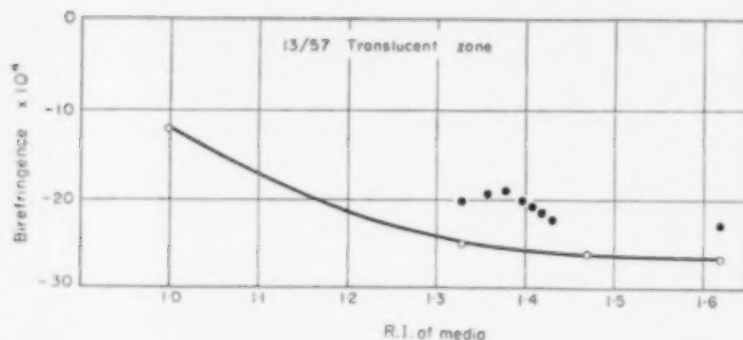


FIG. 9. Observed birefringence at a single point in the translucent zone of a carious lesion imbibed with some of the media listed in Table 1 plotted against the refractive index of the media. Watery media and air (O), alcohols and quinoline (●).

Having assessed the percentage of spaces in the various zones it is now necessary to examine the arrangement of the spaces and in particular the size of the spaces in relation to the molecules which they will admit.

As previously shown, the total amount of spaces in the dark zone at one point examined is approximately 2.5 per cent. It is extremely difficult to assess the amount of spaces accessible to each alcohol used, for each reading reflects the form birefringence of the spaces penetrated by the alcohol and the form birefringence of those spaces which are inaccessible to the alcohol and are therefore still filled with air. However, when quinoline is used, having a refractive index identical with that of enamel, no form birefringence is produced from those spaces filled with quinoline and thus the total form birefringence must be derived from those spaces inaccessible to quinoline which remain filled with air. If the readings shown in Fig. 8 are corrected as already indicated to give an intrinsic birefringence $\times 10^4$ of approximately 30 then the percentage of spaces inaccessible to quinoline can be measured. This is approximately 1.1 per cent or rather less than half of the total percentage of spaces. As *n*-heptanol and *n*-hexanol have the same refractive index the difference in form birefringence in these two media must be largely due to the effects of spaces penetrated by one but not by the other. The higher reading must reflect the amount of air-filled spaces present in the specimen when imbibed with *n*-heptanol, which has the larger molecule, but which are penetrated by *n*-hexanol. By such means it can be shown that approximately 0.1 per cent of additional spaces is penetrated by each succeeding alcohol of smaller molecular size, though between ethanol and methanol there is

probably a much bigger difference possibly of the order of 0.4 or 0.5 per cent. It would be quite wrong to take these figures as an accurate representation of the percentages of spaces present in the dark zone. They are in fact comparative readings and simply show that in the dark zone, which contains possibly 2-4 per cent of spaces, about half of these spaces, or rather more, are large enough to admit the molecules of quinoline, while the other half, or rather less, are quite inaccessible to quinoline even after 24 hr imbibition. Probably slightly more spaces are accessible to *n*-octanol than to quinoline and slightly more again to *n*-heptanol and so on down the series until in ethanol only about one fifth of the spaces are inaccessible to this medium while all the spaces appear to be accessible to methanol, water and air (Table 3).

TABLE 3. APPROXIMATE PERCENTAGE VOLUME OF SPACES ACCESSIBLE AND INACCESSIBLE TO THE VARIOUS MEDIA AT A SINGLE POINT IN THE DARK ZONE OF A CARIOUS LESION OF THE ENAMEL

Medium	Accessible spaces (%)	Inaccessible spaces (%)
Quinoline	1.4	1.1
<i>n</i> -Octanol	1.5	1.0
<i>n</i> -Heptanol	1.6	0.9
<i>n</i> -Hexanol	1.7	0.8
<i>n</i> -Pentanol	1.8	0.7
<i>n</i> -Butanol	1.8	0.7
<i>n</i> -Propanol	1.9	0.6
Ethanol	2.0	0.5
Methanol	2.5 (All)	Nil
Water	2.5 (All)	Nil
Air	2.5 (All)	Nil

The values for the birefringence of one point in normal enamel in the various media are given in Fig. 10. As previously determined, the total percentage of spaces at this point is 0.1 per cent (Table 2). If one now considers the readings in the alcoholic media it can be seen that except for methanol they all give the same value as

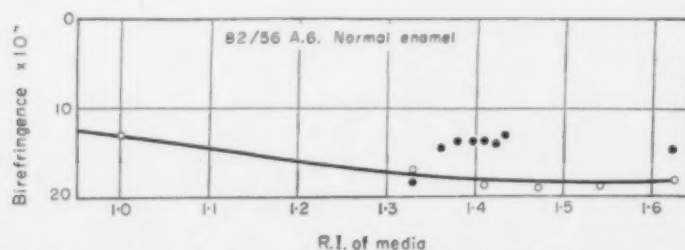


FIG. 10. Observed birefringence at a single point in normal enamel imbibed with some of the media listed in Table 1 plotted against the refractive index of the media. Watery media and air (○), alcohols and quinoline (●).

that for air (Fig. 10). This can only mean that the media have completely failed to penetrate any of the spaces and that the form birefringence produced arises from the total spaces which remain filled with air and thus give the same values as they would when imbibed with air. Again it must be emphasized that these results are only relative and should properly be expressed as showing that normal enamel at this point contained approximately 0.1 per cent of spaces, all of which were too small to admit the molecules of quinoline and the alcohols used, except methanol. They were, however, large enough to admit air, water and methanol.

The total amount of spaces in the body of the lesion as assessed from the corrected form birefringence produced in water is 6.4 per cent at the point observed (Table 2) and, as can be seen from the values in the other media (Fig. 7), all of these spaces were equally accessible to all media used. Thus it can be said that the spaces in the body of the lesion are large enough to admit the molecules of all the media used and, at the point examined (Fig. 7), constitute about 6 per cent of the volume of the enamel.

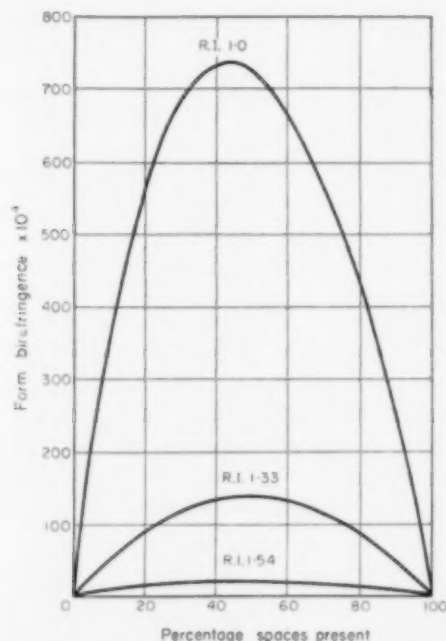


FIG. 11. Graph of total form birefringence which would be produced by various percentages of spaces in enamel when imbibed with media of the refractive indices shown. Based on Wiener's formula. (By kind permission of the Editor of the *British Dental Journal*).

As previously stated, the total amount of spaces present at the point examined in the translucent zone (Fig. 9) was approximately 0.4 per cent. If one takes the readings as shown in Fig. 9, one can also calculate that, of these spaces, approximately 0.1 per

cent were inaccessible to quinoline, that is about one quarter of the total percentage of spaces present at this point. There are, however, certain unusual features about these results. One would have expected greater penetration by the smaller molecules and indeed it is possible that the variation shown here is due to experimental error though it is so consistent as to suggest that there may be an alternative explanation. This is also the only specimen in which methanol did not penetrate the spaces to the same degree as water. For these reasons it is thought that insufficient time has been allowed for full imbibition to occur in these media. For the present, all that can be said is that this point in the translucent zone showed approximately 0.4 per cent of spaces of which at least three quarters were accessible to quinoline.

EXPLANATION OF HISTOLOGICAL APPEARANCES

Normal enamel, seen by transmitted light in quinoline, shows certain structural markings such as the cross striations, the striae of Retzius and the so called inter-prismatic markings (Fig. 13). When seen by ordinary light in an aqueous medium (Thoulet's 1.62) these markings are not apparent (Fig. 14). The findings described above for normal enamel offer a reasonable explanation of this phenomenon. As shown above, normal enamel contains about 0.1 per cent of spaces which are accessible to small molecules such as those of aqueous media but not to the larger molecules of the higher alcohols and quinoline. In the aqueous medium (Thoulet's 1.62) all of these spaces are filled with the medium. As this medium has the same refractive index as the enamel it gives a translucent appearance to the tissue. When the specimen is imbibed with quinoline these spaces are not penetrated by the medium and remain filled with air. Because air (1.0) and enamel (1.62) have such different refractive indices the presence of air-filled spaces in the enamel when seen by ordinary light gives some degree of opacity where the spaces occur. It has been known for many years that the best way of demonstrating the structure in the enamel is to dry the specimen thoroughly in an oven and then mount it directly in Canada balsam. The alternative method of dehydrating in alcohol and passing through xylene before mounting in Canada balsam would also draw out water from the spaces leaving them filled with vapour which would be similar in refractive index to air. The molecules in Canada balsam are very large and would be unable to penetrate the spaces. Thus the appearance of structural markings in the enamel when viewed by ordinary light is caused by the presence at these sites of numerous minute spaces which are not penetrable by the higher alcohols, quinoline and balsam. In these media they remain filled with air and as previously explained produce some opacity at these sites. When normal enamel is seen by polarized light in Thoulet's solution (1.62) some structure is visible because of the variations in the orientation of the crystallites and in the intrinsic birefringence of the different parts of the structure (Fig. 16), but when viewed by polarized light in quinoline or balsam (Fig. 15) the so-called structural elements may become even more obvious because of the form birefringence produced by the tiny spaces filled with air or vapour. The amount of form birefringence thus produced would undoubtedly be small but nevertheless quite sufficient to delineate structure.

The translucent zone of the carious lesion as seen in quinoline and balsam shows as an almost completely transparent zone (Figs. 17, 18) though it is not found in relation to all carious lesions. It shows up by contrast with the normal enamel and its structural markings on one side, and the opacity of the dark zone on the other side. It is also seen in Thoulet's solution (1·62) but is very much more difficult to define as the normal enamel has lost its structural markings and no dark zone is seen in this medium (Fig. 19). From this evidence alone it would seem obvious that the translucence of this zone is caused by the complete imbibition of all spaces with the medium used. This is borne out to a large extent by the findings described above, for at least three quarters of the spaces present were accessible to quinoline and probably also to balsam.

When viewed by polarized light in Thoulet's solution (1·62) (Fig. 21) no form birefringence will be produced in the translucent zone as all the spaces are accessible and the medium has the same refractive index as enamel. In other aqueous media some form birefringence will be produced according to the difference of refractive index between the medium and the enamel, and the translucence of the zone will gradually be lost as the refractive index of the medium becomes more remote from that of enamel when viewed by polarized light. In quinoline (Fig. 20), the large spaces of the translucent zone which are accessible to the medium will produce no form birefringence as the medium has the same refractive index as enamel. But, as has been shown, a few small spaces may remain impenetrable to the molecules of quinoline and produce form birefringence from the air they contain. In normal enamel it has been shown that such small spaces are found chiefly in the structural markings. However, as the translucent zone shows little if any enamel structure the small spaces in this zone are unlikely to be in the structural markings, or structure would be seen.

From the appearances of the translucent zone in Canada balsam (Figs. 22, 23), which are very similar to those found in quinoline (Figs. 24, 25), it must be presumed that the same explanation applies. It is true that Canada balsam (1·54) has a different refractive index from that of enamel and consequently some form birefringence will be produced by the large spaces filled with balsam. However, as the total percentage of spaces is only 0·4–1·0 per cent and the difference in refractive index is effectively very small, very little form birefringence will be produced and the translucent appearance will persist (Figs. 17, 23). This certainly seems to be true if the Canada balsam used is not too viscous but one can imagine that if very treacly balsam or heated pure balsam is used after dehydrating the specimen, the translucence may be reduced or lost because the balsam may fail to penetrate some, if not all, of the large spaces which would remain filled with air.

The dark zone as seen by ordinary light in quinoline or balsam (Figs. 17, 22, 24) shows as an opaque brown or black zone at the border of the carious lesion, separated from the normal enamel at times by the translucent zone. As has already been shown, it contains an appreciable number of extremely small spaces which remain filled with air in both these media. The difference between the refractive index of the air occupying the spaces and that of enamel itself causes strong refractive effects conse-

quent on the dispersion of the light in passage from air to enamel and back again and hence a dark brown opaque colour is produced in this zone. In polarized light in quinoline or balsam (Figs. 23, 25) the difference in refractive index between the contents of the spaces and the enamel causes the production of a high degree of form birefringence which reduces or cancels the negative intrinsic birefringence of the enamel and results in the so called positive zone, which is identical with the dark zone. In aqueous media, methanol and air, when viewed by polarized light, form birefringence is produced but this is only the normal amount produced by a medium which enters all the spaces and which varies according to the refractive index of the medium. This is reflected in the imbibition curve (Fig. 8).

The body of the lesion contains only spaces which are accessible to all media and therefore appears translucent in ordinary light when the refractive index of the imbibing medium is similar to that of enamel whether the molecules of the medium are large or small, e.g. quinoline and Thoulet's medium (1.62) (Figs. 18, 19). When imbibed with other media with refractive indices different from that of enamel then some degree of opacity will be produced in transmitted light. In polarized light the body of the lesion shows form birefringence which is wholly dependent on the refractive index of the imbibing medium as this zone contains only spaces which are large enough to accommodate the molecules of all the media (Figs. 20, 21, 23, 24). However, in specimens mounted in Canada balsam (1.54) there is the additional possibility that the balsam may fail to penetrate some of the spaces because of its physical state, as described for the translucent zone. This may result in increased opacity in ordinary light and increased form birefringence in polarized light (Figs. 22, 23, 26, 27).

THE ORIGIN OF SPACES IN THE CARIOUS LESION

From the evidence given there appears to be a pattern of spaces within normal and carious enamel (Tables 2, 3) which causes the histological appearances found in these areas in ground sections. Normal enamel has been found to contain a few small spaces which are accessible only to water, air and methanol though this does not preclude the existence of other spaces. In the development of the translucent zone during the attack of caries these small spaces may persist or may be lost, but the major feature is the production of a considerable amount of large spaces accessible even to the large molecules of quinoline and *n*-octanol. The dark zone contains even more of these large spaces than does the translucent zone but it is dominated by an almost equally large amount of minute spaces which are inaccessible to quinoline and to a less degree to other media which have molecular sizes intermediate between that of quinoline and methanol. In the body of the lesion only large spaces accessible to quinoline are found.

It is quite obvious that the large number of small spaces in the dark zone constitutes a new development in the carious lesion for they cannot be derived from the large spaces found in the translucent zone. They are too small to have been produced by the removal of organic material for they are much smaller than any organic molecule likely to occur in the matrix of the enamel. Thus they must be regarded as

manifestations of demineralization. This is consistent with the evidence from micro-radiography (DARLING, 1958). The succession of sizes in these spaces indicates that they are gradually enlarged so that by the time the body of the lesion is reached all the spaces are large enough to admit quinoline.

The nature of the translucent zone is less easily explained. From the evidence given it contains a much higher amount of spaces than normal enamel but most of these spaces, if not all, are large enough to admit quinoline. The apparent presence of small spaces which are inaccessible to quinoline may indicate that such spaces are actually present or that they are an expression of too short a time for imbibition of the medium. In the latter case they are really accessible to quinoline but only slowly. If they are actually too small to admit quinoline and the higher alcohols they may represent a stage in the development of the large spaces or they may be an overlay from the early beginnings of the dark zone or a persistence of the small spaces from the normal enamel supplemented by an overlay from the dark zone. They are unlikely to be left over from normal enamel as the structural markings which they produce in normal enamel are lost in the translucent zone. If they are a stage in the production of large spaces then the process is similar to the production of large spaces in the body of the lesion from small spaces in the dark zone. In such circumstances one would expect a preponderance of these small spaces near the junction between the normal enamel and the translucent zone where they have recently been formed and have not yet had time to enlarge. This is not found. The more probable explanation is that the large spaces characteristic of the translucent zone arise as large spaces with or without some overlay of small spaces from the dark zone. The fact that translucent zones are not always seen around the carious lesion in enamel suggests a variable degree of overlay from the dark zone which at times nullifies the effect of the large spaces and causes a loss of translucence.

The origin of the large spaces in the translucent zone is by no means clear. Obviously they must arise by loss of tissue from the enamel and if the structural markings of the enamel are lost it seems reasonable to suppose that the large spaces have engulfed the tiny spaces which have been shown to be the cause of the structural markings as seen by transmitted light in normal enamel imbibed with quinoline. This could arise by the enlargement of the tiny spaces with loss of mineral or loss of organic matrix. However, such zones can be produced by the loss of organic material alone without the loss of minerals from the enamel. This is shown by the production of translucent zones caused by the action of cold, 1,2-diamino-ethane (ethylene-diamine) on normal enamel (DARLING and MORTIMER, 1959). Nevertheless it remains possible that these large spaces might be produced by the loss of mineral only or by the loss of mineral and organic material.

Acknowledgements—The authors wish to express their appreciation of the financial assistance provided by the Medical Research Council for the support of K. V. MORTIMER and D. F. G. POOLE.

They would also like to acknowledge the considerable technical assistance given by B. G. H. LEVERS and other members of the laboratory staff.

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PLATE I

FIG. 12. Models of the molecules of *n*-propanol (left) and *iso*-propyl alcohol (right) to show the difference in form in spite of similar chemical composition.

FIG. 13. Ground section of normal enamel mounted in quinoline (1·62) and seen by transmitted light. $\times 320$.

FIG. 14. Ground section of normal enamel showing approximately the same field as in Fig. 13 mounted in Thoulet's solution (1·62) and seen by transmitted light. $\times 320$.

FIG. 15. Ground section of normal enamel showing the same field as in Fig. 13 mounted in quinoline and seen by polarized light in the 45° position. $\times 320$.

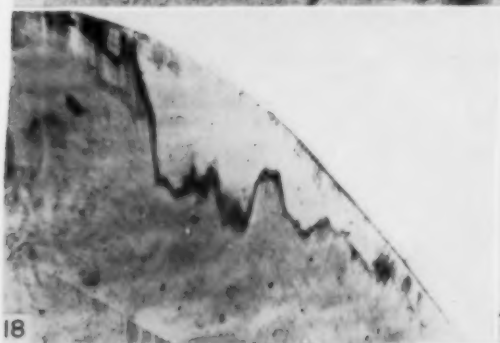
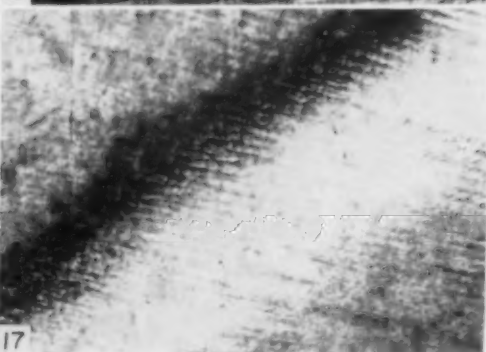
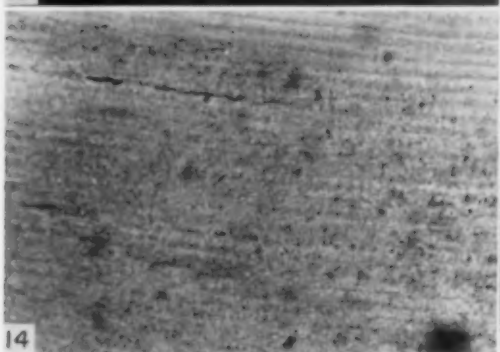
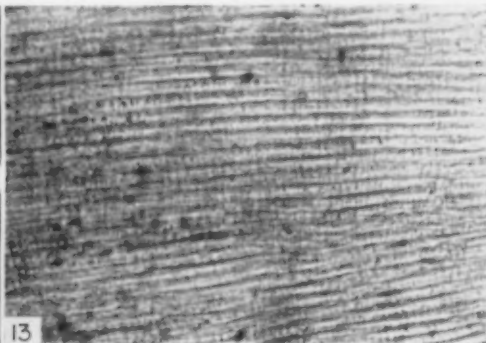
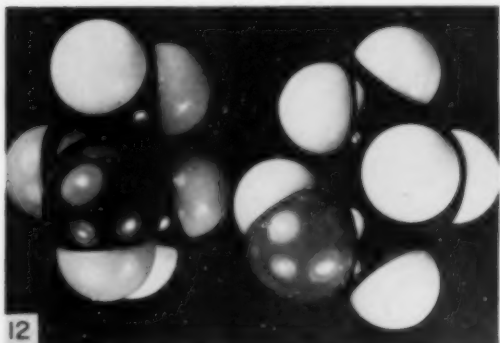
FIG. 16. Ground section of normal enamel showing the same field as Fig. 14 mounted in Thoulet's solution (1·62) and seen by polarized light in the 45° position. $\times 320$.

FIG. 17. Carious enamel at the inner border of a lesion mounted in Canada balsam and seen by transmitted light. The translucent zone can be seen showing very few structural markings in contrast to the normal enamel on its right. $\times 280$.

FIG. 18. Ground section of enamel caries imbibed with quinoline (1·62) and seen by transmitted light. $\times 24$.

FIG. 19. Same specimen as in Fig. 18 imbibed with Thoulet's solution (1·62) and seen by transmitted light. $\times 24$.

MOLECULAR SIEVE BEHAVIOUR OF NORMAL AND CARIOUS HUMAN DENTAL ENAMEL



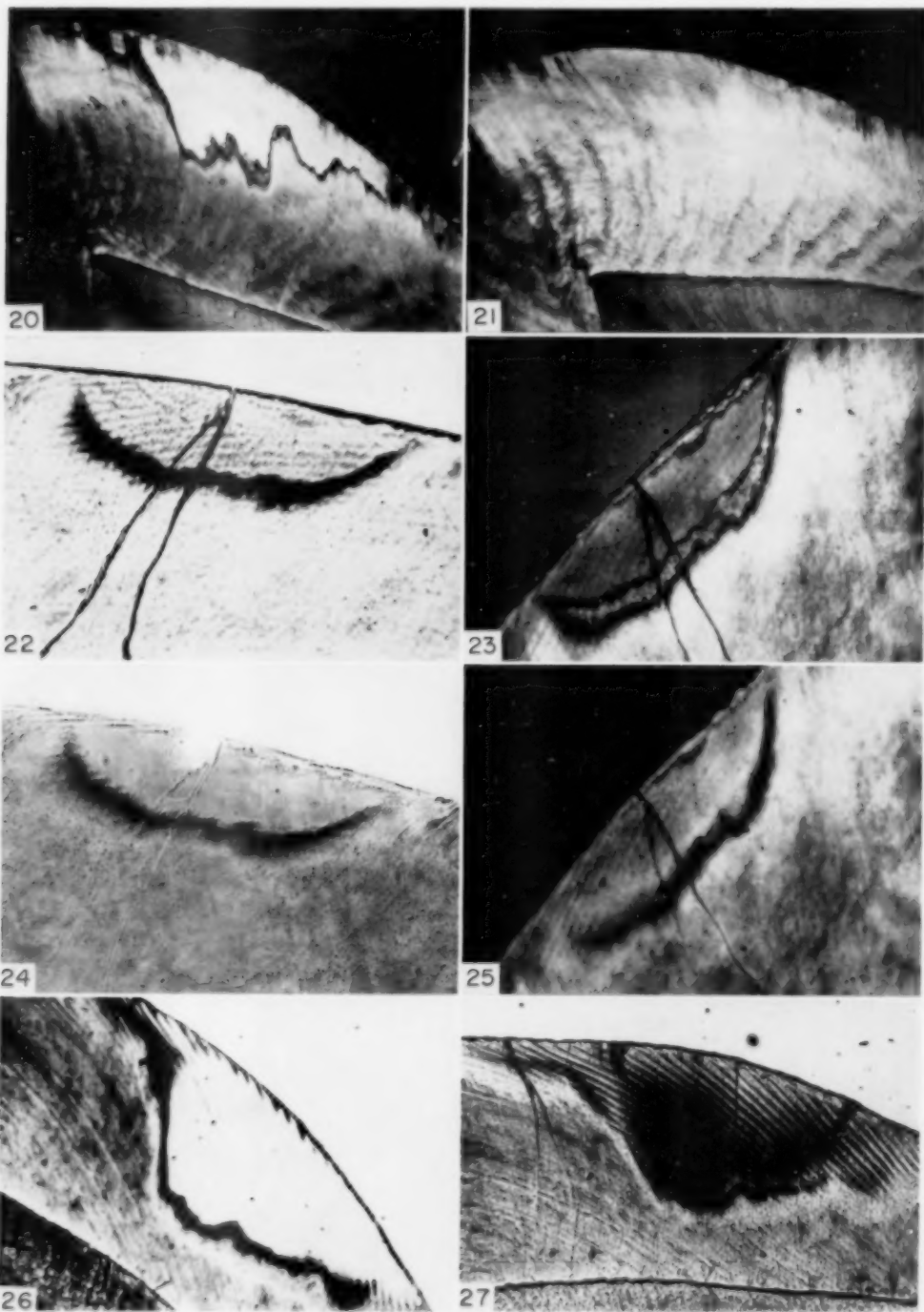


PLATE 2

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PLATE 2

FIG. 20. Same specimen as in Fig. 18 imbibed with quinoline (1·62) and seen by polarized light in the 45° position. $\times 24$.

FIG. 21. Same specimen as in Fig. 18 imbibed with Thoulet's solution (1·62) and seen by polarized light in the 45° position. $\times 24$.

FIG. 22. Ground section of enamel caries mounted in relatively thin Canada balsam and seen by transmitted light. $\times 80$.

FIG. 23. Specimen and mountant as in Fig. 22 seen by polarized light in the 45° position. $\times 80$.

FIG. 24. Specimen as in Figs. 22 and 23 imbibed with quinoline (1·62) and seen by transmitted light. $\times 80$.

FIG. 25. Specimen as in Figs. 22-24 imbibed with quinoline (1·62) and seen by polarized light in the 45° position. $\times 80$.

FIG. 26. Ground section of enamel caries imbibed with quinoline and seen by transmitted light. $\times 36$.

FIG. 27. Same specimen as in Fig. 26 mounted in thick Canada balsam and seen by transmitted light. $\times 36$.

CITRATE IN MINERALIZED TISSUES—IV THE RELATION OF VITAMIN D INTAKE AND CALCIUM NUTRITION TO THE CITRATE CONTENT OF THE RAT FEMUR

R. L. HARTLES and A. G. LEAVER*

Biochemistry Department, School of Dental Surgery,
University of Liverpool, England

Abstract—Hypervitaminosis-D caused a large increase in the citrate content of the rat femur on both normal and low intakes of calcium. A diet low in calcium (<0.03 per cent w/w) resulted in a lowering of the calcium and phosphorus content of the bone when the animal received either a normal or excessive (250×) intake of vitamin D. A diet with excessive vitamin D and a normal calcium content resulted in a slight fall in the calcium content of the femur and a greater fall in the phosphorus content. The calcium/phosphorus ratio was significantly higher when the intake of vitamin D was excessive. The implications of these results are discussed.

INTRODUCTION

PREVIOUSLY it has been shown (HARTLES and LEAVER, 1961) that a purified diet low in calcium (<0.03 per cent) but adequate in vitamin D results in a 50 per cent elevation of bone citrate above the normal value. A diet deficient in vitamin D but adequate in calcium causes a small depression in the value of bone citrate. A diet deficient in both calcium and vitamin D results in a bone citrate value which is only half the normal figure.

The experiments now reported were designed to investigate the effect of hypervitaminosis-D on the composition of the bone. Rats were maintained on four diets, a control diet, a diet deficient in calcium, a diet with a normal calcium content but containing 250 times as much vitamin D as the control, and a diet deficient in calcium containing 250 times as much vitamin D as the control diet. All diets contained adequate phosphate.

The findings were that an excessive intake of vitamin D irrespective of the intake of calcium caused the citrate content of the rat femur to be almost doubled. Hypervitaminosis-D resulted in the raising of the calcium/phosphorus ratio of the bones both on the low and normal calcium diets. Of the animals which received a high intake of vitamin D those on the low calcium diet grew better than those on the normal calcium diet.

EXPERIMENTAL

Animals. Forty month-old rats (20 male, 20 female) were distributed between four equal groups.

Diets. The basic diets and salt mixtures were the same as those used in earlier experiments (HARTLES and LEAVER, 1961).

* Nuffield Dental Research Fellow.

HS7—control diet, contained 0.56 per cent (w/w) calcium, each 960 g of diet contained 200 μ g ergocalciferol.

HS8—low calcium diet, contained <0.03 per cent calcium, otherwise as HS7.

HS11—As HS7 except that each 960 g contained 50 mg ergocalciferol.

HS12—As HS8 except that each 960 g contained 50 mg ergocalciferol.

The diets and deionized water were given to the animals in unrestricted amounts.

Group A received diet HS7; Group B, HS8; Group C, HS11; and Group D, HS12.

Housing. Five rats were kept in a wire mesh cage with $\frac{5}{16}$ in. mesh floor, and were weighed twice weekly. The environmental temperature was maintained between 70 and 75°F.

Duration of experiment. The animals were maintained on their respective diets for 8 weeks; they were then killed by ether inhalation.

Analytical methods. The femora were prepared and analysed for calcium, phosphorus, nitrogen and citrate as described previously (HARTLES, 1951a,b; HARTLES and LEAVER, 1960, 1961).

RESULTS

Growth of animals. The average weekly gains in weight over the experimental period are shown in Fig. 1. It is interesting to note that the animals of Group C grew less well than those of Group D. It appears that a low calcium intake protects the animal to some extent from the deleterious effects of hypervitaminosis-D.

TABLE 1. THE EFFECT OF NORMAL AND HIGH INTAKES OF VITAMIN D AND NORMAL AND LOW INTAKES OF CALCIUM ON THE PERCENTAGE COMPOSITION OF THAT PORTION OF THE RAT FEMUR OF S.G. ≥ 1.5

	Group A. Normal Ca, normal vit. D	Group B. Low Ca, normal vit. D	Group C. Normal Ca, high vit. D	Group D. Low Ca, high vit. D
Calcium	24.3 \pm 0.29	20.2 \pm 0.16	23.3 \pm 0.21	20.4 \pm 0.17
Phosphorus	12.1 \pm 0.71	10.0 \pm 0.11	10.6 \pm 0.09	9.6 \pm 0.14
Calcium/ phosphorus ratio	2.01 \pm 0.02	2.02 \pm 0.02	2.18 \pm 0.03	2.13 \pm 0.02
Nitrogen	3.7 \pm 0.03	5.7*	4.4 \pm 0.05	5.6*
Citrate	0.53 \pm 0.009	0.68 \pm 0.015	0.96 \pm 0.022	0.97 \pm 0.015
Citrate/g calcium (mg)	21.7 \pm 0.37	34.0 \pm 0.92	41.2 \pm 0.97	47.6 \pm 0.98

* Average value of two pooled samples, each from five animals.

Analytical data. The results are given in Table 1.

Calcium. In the animals of Groups B and D the calcium content of the bone was significantly lower than that of the control animals. It seems therefore that irrespective

of vitamin D intake, if the calcium intake was low the calcium content of the bone was lowered by a similar amount. In Group C animals which received a diet containing normal amounts of calcium the effect of hypervitaminosis-D was to cause a smaller but significant fall in the calcium content of the bone ($P<0.005$).

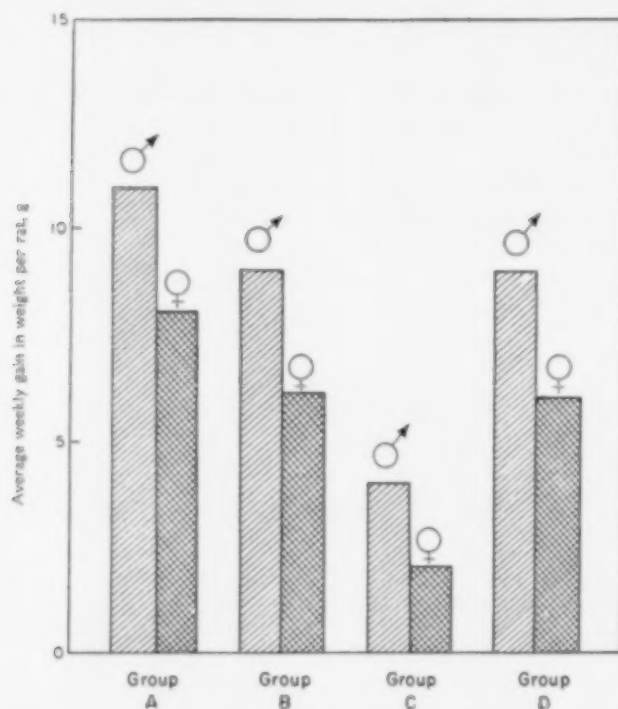


FIG. 1. Average weekly gains in weight per rat (g). Group A, normal calcium, normal vitamin D. Group B, low calcium, normal vitamin D. Group C, normal calcium, high vitamin D. Group D, low calcium, high vitamin D.

Phosphorus. The phosphate content of the bone was significantly lower in Groups B, C and D when compared with the control group A ($P<0.005$). The greatest fall was in Groups B and D. Thus a low calcium intake depressed the bone phosphate when the vitamin D intake was either normal or excessive but the depression was greater in hypervitaminosis ($P<0.005$).

Calcium/phosphorus ratio. There was no significant difference between the ratios for Groups A and B, or between Groups C and D. There was a significant difference between the calcium/phosphorus ratios when the animals of Groups C and D were compared with those of Groups A and B. Hypervitaminosis-D increased the calcium/phosphorus ratio by diminishing the phosphate level to a relatively greater extent than the calcium, irrespective of the level of calcium intake.

Nitrogen. There was an obvious increase in the nitrogen content of the bone in Groups B and D animals. The increase in Group C was also significant but less marked than in Groups B and D.

Citric acid. There was an obvious increase in the citrate content of the bones of the Group B animals compared with those of Group A, and with those of Groups C and D compared with Group A and Group B. There was no significant difference between the four values for Groups C and D.

Citric acid/calcium ratio. When the results were expressed in this way, as mg citric acid per g calcium, there were highly significant differences between all four groups.

Observations on the size of the femora. The animals of Groups B and D had small undermineralized bones with wide medullary areas. In Group C the bones were small but hard and highly mineralized and the medullary area was narrow. It must be emphasized before discussing any implications of these results that they refer to that portion of the bone of S.G. ≥ 1.5 . Not only did the quality of the bone vary but the quantity of mineralized tissue varied in the different groups. The average weight of the fraction of bone of S.G. ≥ 1.5 obtained from each pair of femora was as follows: Group A, 190 mg; Group B, 54 mg; Group C, 162 mg; Group D, 65 mg.

DISCUSSION

In a previously described experiment (HARTLES and LEAVER, 1961) data were presented showing the effect of avitaminosis-D in the presence and absence of dietary calcium on the bone composition of the rat. It is pertinent to discuss the present results together with some of the earlier ones. For this purpose the results have been re-arranged in Table 2.

On examining the data presented in Table 2 it can be seen that the absence of calcium in the diet had a greater effect on the mineral content of the bone formed than did variation in the dietary level of vitamin D. Nevertheless in the presence of adequate calcium the conditions of avitaminosis and hypervitaminosis had some effect in reducing the calcium and phosphorus content of the bone when compared with the controls. These differences were highly significant ($P < 0.005$), and are shown in Figs. 2 and 3.

When the intake of vitamin D was normal or when it was absent from the diet, there was no significant difference in the calcium/phosphorus ratio at low or normal dietary levels of calcium. Thus in the absence of the vitamin the mineralized matrix contained less calcium and phosphorus, but the relative composition of the mineral deposited as indicated by the calcium/phosphorus ratio was not significantly changed. In hypervitaminosis-D however not only were the amounts of mineral deposited less than in the controls, the calcium/phosphorus ratios were significantly higher than in the normal animals. There was also a large increase in the citrate content of the bone when the intake of vitamin D was excessive. These findings are not inconsistent with the view that in hypervitaminosis-D, irrespective of calcium intake,

citrate can replace some of the phosphate in the bone crystal, thus lowering the calcium/phosphorus ratio.

TABLE 2. THE CALCIUM, PHOSPHORUS, CITRATE AND NITROGEN COMPOSITION OF THAT PORTION OF THE RAT FEMUR OF S.G. ≥ 1.5 , AT VARYING LEVELS OF VITAMIN D INTAKE, AND AT NORMAL AND LOW LEVELS OF DIETARY CALCIUM
Each result is the mean \pm S.E.M. of ten observations

Vitamin D (ergocalciferol) added to each 960 g of diet		None*	200 μ g	50 mg
Calcium (%)	(i)	19.9 \pm 0.18	20.2 \pm 0.16	20.4 \pm 0.17
	(ii)	23.5 \pm 0.12	24.3 \pm 0.29	23.3 \pm 0.21
Phosphorus (%)	(i)	9.7 \pm 0.09	10.0 \pm 0.11	9.6 \pm 0.14
	(ii)	11.6 \pm 0.06	12.1 \pm 0.17	10.6 \pm 0.09
Ca/P ratio	(i)	2.04 \pm 0.02	2.02 \pm 0.02	2.13 \pm 0.02
	(ii)	2.03 \pm 0.01	2.01 \pm 0.02	2.18 \pm 0.03
Citrate (%)	(i)	0.24 \pm 0.014	0.68 \pm 0.015	0.97 \pm 0.015
	(ii)	0.46 \pm 0.010	0.53 \pm 0.009	0.96 \pm 0.022
Citrate/g calcium (mg)	(i)	12.2 \pm 0.67	34.0 \pm 0.92	47.7 \pm 0.98
	(ii)	19.5 \pm 0.48	21.7 \pm 0.37	41.2 \pm 0.97
Nitrogen (%)	(i)	5.9 \pm 0.05	5.7†	5.6†
	(ii)	3.9 \pm 0.03	3.7 \pm 0.03	4.4 \pm 0.05

(i) Calcium in diet <0.03 per cent (w/w); (ii) Calcium in diet 0.56 per cent (w/w).

* Results taken from HARTLES and LEAVER (1961).

† Average of two pooled samples, each from five animals.

Citrate content of bone

The relationship of the parathyroid hormones and vitamin D to calcium metabolism is intriguing and baffling. Early suggestions that vitamin D had a direct effect upon the hormone are unlikely since vitamin D can raise the blood calcium, i.e. mobilize skeletal calcium in the parathyroidectomized animal, but only if the calcium/phosphorus ratio of the diet is high (TWEEDY *et al.*, 1939). Evidence suggesting that both vitamin D and parathyroid hormone influence calcium metabolism independently but adjutantly has been gathered and correlated by NEUMAN and NEUMAN (1958). They elaborate the hypothesis that both vitamin D and the parathyroid hormone exert their effect by stimulating the production of citric acid at different points in the glycolytic metabolic pathways.

We have confirmed our earlier observation (HARTLES and LEAVER, 1961) that with a normal vitamin D intake the citrate concentration in the rat femur is much greater when the animal received a diet very low in calcium. There was an increase

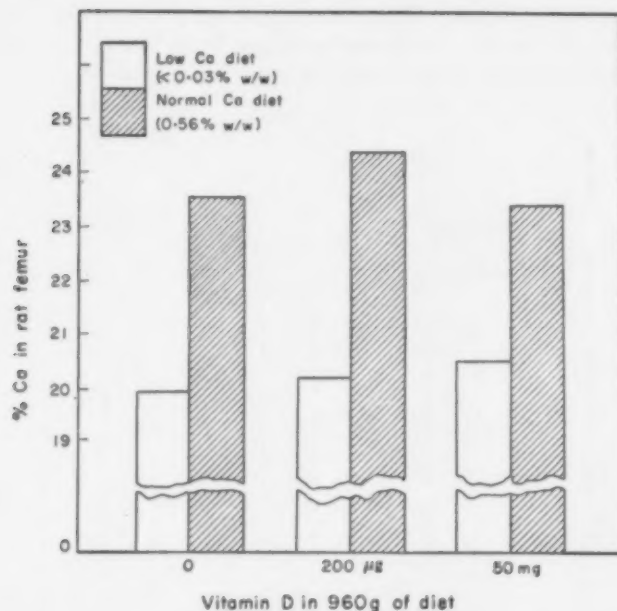


FIG. 2. The calcium content of that portion of the rat femur of a specific gravity of not less than 1.5 at varying dietary levels of vitamin D and calcium.

both in the absolute amount of citrate and in the citrate/calcium ratio. When the dietary content of vitamin D was raised to a level 250 times the control value there was a further large increase in bone citrate (Fig. 4). When these results are expressed as a citrate/calcium ratio, it is found that there was a significantly greater increase when the diet was low in calcium than when the calcium intake was normal (Fig. 5).

Thus in our experiments both the highest and the lowest levels of bone citrate were observed in animals which had been deprived of an adequate calcium intake. The variable factor was the vitamin D content of the diet.

If the diet contained adequate calcium the synthesis of bone citrate was sensitive to lack of vitamin D, but its production was enhanced by excessive doses of the vitamin both in the presence and absence of calcium in the diet. It can be concluded therefore that when the necessity to mobilize calcium from the bones to the serum is greatest, vitamin D exerts its greatest action on the synthesis of bone citrate.

In the absence of both vitamin D and adequate calcium the bone formed has its lowest content of citrate (HARTLES and LEAVER, 1961). After 8 weeks on this diet the animals exhibited signs of convulsive tetany. Thus in the absence of adequate vitamin D and when the intake of calcium was low, the parathyroids were unable to mobilize the calcium in the skeleton to maintain the serum calcium concentration at a sufficient level to prevent the onset of tetany. If the diets contained normal or excessive amounts of vitamin D no signs of tetany were produced even when the

intake of calcium was very low. Thus tetanic signs were associated with low bone citrate and hence a low citrate concentration in the fluids in contact with the bone crystals. HOWARD (1956) has emphasized that it is the equilibrium at the solid/fluid

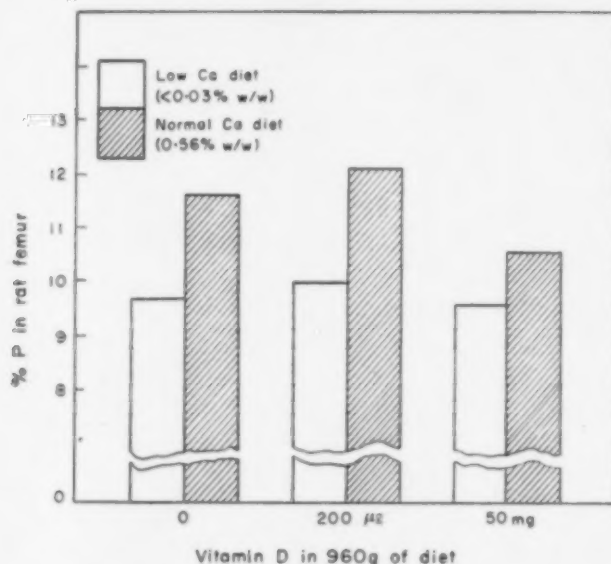


FIG. 3. The phosphorus content of that portion of the rat femur of a specific gravity of not less than 1.5 at varying dietary levels of vitamin D and calcium.

interface in the bone itself that governs the level of circulating calcium. Our results lend support to the view that the production of citrate in bone is one of the important factors in the mobilization of minerals from the skeleton. There is some evidence to suggest that the citrate in mineralized tissues exists in at least two forms (HARTLES and LEAVER, 1960; LEAVER, EASTOE and HARTLES, 1960; TAYLOR, 1960; BODEN, 1960). One fraction appears to be associated with a peptide, another is released apparently as uncombined citrate on demineralization with dilute hydrochloric acid or with ethylenediaminetetra-acetate. It could be that the fraction associated with the peptide is not readily available as a chelator of calcium for the maintenance of homeostasis, and that the citrate exists mainly in this form when the dietary intakes of calcium and vitamin D are very low. It could further be postulated that there is a "constant" fraction and a "variable" fraction of bone citrate. The "constant" fraction would be of the order of 12–14 mg citrate/g calcium. The lowest bone citrate we have ever observed was 12.3 mg/g calcium. CARLSSON and HOLLUNGER (1954) reported comparable low values of 13.9 mg/g calcium. We have not found reports of any lower values, and suggest that bone citrate does not fall below this figure which represents the "constant" fraction. Bone containing this quantity of citrate

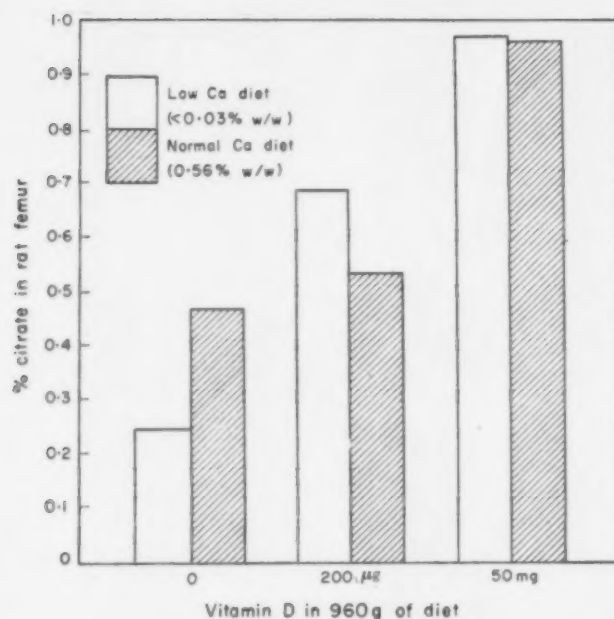


FIG. 4. The citrate content of that portion of the rat femur of a specific gravity of not less than 1.5 at varying dietary levels of vitamin D and calcium.

appears unable to contribute to the maintenance of an adequate concentration of serum calcium, whereas bone containing similar amounts of calcium (low calcium diet, normal vitamin D) but 34.6 mg citrate/g calcium prevents the onset of tetany. If it is assumed that the "variable" fraction is numerically equal to the difference between the total bone citrate and the hypothetical "constant" fraction we obtain the figures given in Table 3.

TABLE 3

Calcium in diet (%)	"Variable" fraction (mg citrate/g calcium)		
	No vitamin D	Normal vitamin D	High vitamin D
<0.03	0	22.3	35.4
0.56	7.2	9.5	28.9

We are aware that in postulating a "constant" and "variable" fraction of bone citrate we may only be playing with figures, nevertheless the concept is not incompatible with the properties and behaviour of bone citrate. The "variable" fraction

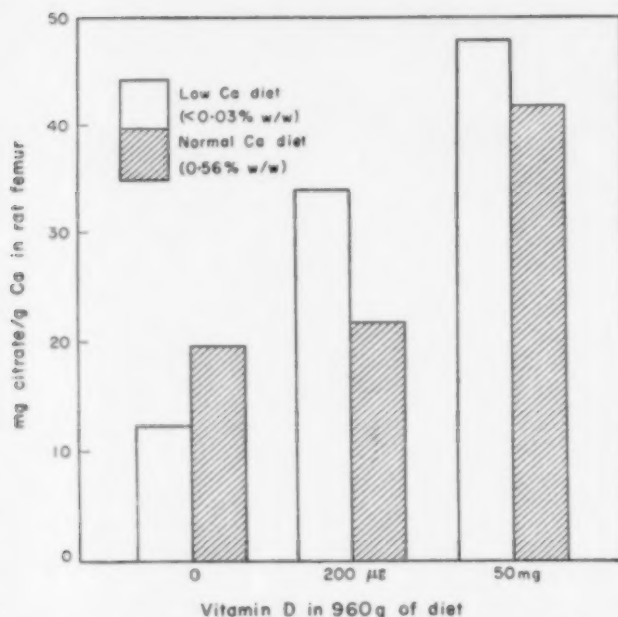


FIG. 5. The ratio of mg citrate per g calcium of that portion of the rat femur of a specific gravity of not less than 1.5 at varying dietary levels of vitamin D and calcium.

is small when calcium intake is normal and when the intake of vitamin D is not excessive. In conditions of stress such as extreme calcium lack, citrate production in bone is vastly increased only if vitamin D is available. It is concluded therefore that the synthesis of bone citrate is directly governed by vitamin D, but that an adequate intake of dietary calcium will lessen the effect of the vitamin.

Interrelation of vitamin D and parathyroid hormone

An attractive and ingenious hypothesis has been postulated by NEUMAN and NEUMAN (1958) in an attempt to correlate the actions of the vitamin and hormone. We have already commented on this (HARTLES and LEAVER, 1961) and our present results do nothing to resolve the dilemma. The hypothesis suggests that parathyroid hormone and vitamin D influence different co-factors in carbohydrate metabolism. In brief, the hormone inhibits oxidative reactions requiring TPN and the vitamin stimulates reactions requiring DPN or cocarboxylase. There is some *in vitro* evidence to support these views. Vitamin D will therefore tend to encourage the production of citrate (DPN dependent), parathyroid hormone will hinder its further oxidation (TPN dependent) and also will encourage glycolysis (DPN dependent) and the production of pyruvate by inhibiting the TPN dependent pentose oxidation shunt. Thus in the absence of vitamin D the hormone will not actually cause an increase in citrate production, but it will encourage the production of pyruvate which, in the presence of the vitamin, can be converted to citrate. In other words the hormone alone

cannot stimulate citrate production whereas vitamin D alone can. Together they will exert the maximum effect.

We have no direct evidence to present, but it can fairly be assumed that in the animals on low calcium diets (Table 2) the parathyroids would in general be stimulated more than in the animals receiving an adequate calcium intake. In the presence of vitamin D the animals produced more citrate when the calcium intake was low, i.e. when the parathyroids were stimulated, than when the calcium intake was normal. This is in accord with the Neuman's hypothesis. The anomalous result however is that in the absence of the vitamin less citrate is formed on a low calcium diet (parathyroid stimulated) than on the normal calcium intake. If the hypothesis is correct the level of citrate should be at least no greater in the presence of calcium.

This is a fascinating problem and we intend to study the influence of varying the phosphate intake on the production of bone citrate in the presence of different amounts of vitamin D.

Acknowledgements—The authors thank Mr. R. P. WILLIAMS for his care and maintenance of the animals; Mr. J. S. BAILLIE for designing the figures; Messrs. J. BIBBY and Sons Ltd. for the gift of groundnut oil; and Messrs. TATE and LYLE Ltd. for the gift of icing sugar.

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THE RESULTS OF 6½ YEARS OF ARTIFICIAL FLUORIDATION OF DRINKING WATER IN THE NETHERLANDS THE TIEL—CULEMBORG EXPERIMENT

O. BACKER DIRKS, B. HOUWINK and G. W. KWANT

Organization for Health Research T.N.O., Laboratory of Microbiology,
University of Utrecht, Netherlands

Abstract—In order to assess the effect of water fluoridation on dental caries under Dutch living conditions (food, water consumption, etc.) the drinking water of Tiel was fluoridated since 1953 at 1.1 mg/l. The nearby city of Culemborg served as control (± 0.1 mg natural fluoride per litre of water). The principal study groups contained each second year the 11–15 year old children from both cities. Approximal caries was estimated from radiographs only, caries of occlusal and free smooth surfaces in a clinical examination. Both methods were standardized as far as possible. The results after 6½ years of water fluoridation show an important caries inhibiting effect on caries of approximal and free smooth surfaces. The inhibition of occlusal cavities is (at the present time) far less marked. The favourable effect of this water fluoridation is in many respects similar to the results of American studies after the same interval.

INTRODUCTION

THE WELL-KNOWN investigations on artificial water fluoridation in America which started in 1945 (Grand Rapids, Newburgh, Brantford) and 1946 (Evanston) demonstrated the importance of this measure in the prevention of dental caries (ARNOLD *et al.*, 1956; AST *et al.*, 1956; HILL, BLANEY and WOLF, 1957; BROWN, McLAREN and POPHOVE, 1960). The numerous studies of the last three decades on the effect of fluorides strongly point to the fact that water fluoridation does not endanger the health of the consumer (ORGANISATION MONDIALE DE LA SANTÉ, 1958; HEALTH COUNCIL, 1960; MUHLER and HINE, 1960).

Notwithstanding these studies, the Organization for Health Research T.N.O. in 1951 considered that they were an insufficient basis for fluoridation of the drinking water in the Netherlands. The differences both in dietary habits—which strongly influence caries activity—and in the consumption of drinking water—which determines the fluoride-uptake—made it impossible to apply with confidence the results of the American studies to the Netherlands.

For these reasons it was decided to carry out a study in the Netherlands.

A sociological investigation of a large number of municipalities was carried out in order to find the most suitable pair of cities.

Attention was given to population structure, site, size (above 15,000 inhabitants), migration and water composition, and two cities were selected which were as equal as

possible in these and other respects. Tiel and Culemborg, situated between the rivers Rhine and Maas at a distance of 10 miles from each other, were chosen for the study.

In March 1953 the drinking water in Tiel was fluoridated at a level of 1.1 mg/l. Culemborg with a fluoride concentration of 0.10 mg/l was to serve as control.

METHODS OF CARIES EXAMINATION

Generally four main types of dental caries lesions are distinguished, i.e. lesions of fissures and pits, of the approximal surfaces, of the free smooth surfaces (buccal and lingual surfaces) and of the cervices of the teeth.

This differentiation is more than a purely anatomical and clinical classification. The intra-patient correlation of these types of caries is generally poor (BARR, DIODATI and STEPHENS, 1957) and lends weight to the supposition that the systemic and dietary factors leading to the initiation of caries are not the same for these four types of lesions. Moreover, it is known that these different types of caries are not influenced to an equal extent by fluorides.

The grouping together for statistical purposes of these different caries forms may result in a distorted picture and in the loss of valuable information. It was therefore decided to evaluate the various forms separately.

As approximal caries is the most important one from the therapeutic point of view, our attention was directed in the first place to the evaluation of this type.

Caries of the approximal surfaces

For approximal caries the clinical examination using mirror and explorer was completely abandoned, because of its poor accuracy which makes it almost impossible to standardize diagnosis (GREEN and WEISENSTEIN, 1960; BACKER DIRKS and VAN AMERONGEN, 1953). Furthermore with this method it is impossible to exclude variation in the standard, either during an examination or between yearly examinations and, what is even worse, there is no way of determining the extent of this change in standard.

Approximal caries was exclusively diagnosed from radiographs as described elsewhere (BACKER DIRKS, VAN AMERONGEN and WINKLER, 1951; BACKER DIRKS and KWANT, 1954). All radiographs were made with an apparatus which ensures the correct position of the film in the mouth and of the X-ray beam on the film, so as to ensure reproducible radiographs. All exposures were made at a constant primary voltage and the development of the films was also standardized. The caries examination required two bitewing-radiographs for the posterior teeth and three for the upper anteriors. In general, no radiographs were made of the lower anterior teeth. The exceptional skewness of the frequency distribution of the approximal lesions of the lower anterior teeth makes these data of questionable value. For this reason the lower anterior teeth were only examined in some age groups.

Since all radiographs were made in duplicate to ensure reliability, ten pictures were required. Owing to the standardization of the technique the preparation of the ten radiographs took less than 5 min.

The caries diagnosis made from the radiographs was standardized as far as possible. Four observers (two dentists and two technicians) took part in the evaluation of the radiographs. These observers were given special training in reading radiographs for which carefully checked series of previous films were used as a standard. To avoid and check a possible shift in standard of examination, the observers were regularly required to evaluate a standard set of films between their normal work, i.e. generally after evaluating the radiographs of forty-five patients or less.

Each radiograph was examined independently by one dentist and one technician, and the average score of the two estimations was used in all calculations. After the examination of the radiographs of fifteen subjects the combination of the two observers was changed. The analysis of the results of the four examiners proved the consistency of their examinations. Each examiner evaluated the same number of radiographs from Tiel and from Culemborg without knowledge of the origin of the films.

The approximal carious lesions were classified with regard to the degree of penetration in the direction of the pulp. As the assessment was made from a radiograph it represents the projection and not the actual extent in depth of the lesion. In the case of a lesion extending over a large area or in rotated teeth this will sometimes have resulted in an inaccurate assessment.

Caries I indicates a carious lesion which is limited to the enamel. Caries stages II, III and IV indicate respectively a lesion which has penetrated the dentine, which extends from the enamel-dentine junction halfway to the pulp, or which has reached the pulp. Category V indicates a filling (including crowns, etc.). In general, the caries is indicated as caries I-V, i.e. all lesions and fillings, or as caries II-V, by which is meant those lesions which have already penetrated the dentine. The latter are the lesions which should have been filled, if they are not yet treated. Each surface was separately scored so that all calculations could be carried out per surface and per child.

Since all radiographs were evaluated twice, the number of deviating estimations can be counted and used as a measure for the error of observation. The standard deviation of the evaluation of the percentage of carious surfaces in a group of ± 100 children is approximately 0.5 per cent, or about one tenth of the standard deviation originating from the scattering of the sample.

The great advantage of the method employed was also that it made a blind evaluation possible: the radiographs made in Tiel and Culemborg were put into unlabelled envelopes, and examined at random.

Pit and fissure caries and caries of the free smooth surfaces

During the clinical inspection of the mouth the teeth present, enamel hypoplasia and hypocalcifications and dental fluorosis, the type of occlusion, the condition of the mucous membranes, the state of oral hygiene, etc., were recorded. After this inspection, selected age classes were examined for pit and fissure caries and for buccal smooth surface lesions.

The method of examination of the pits and fissures has already been published (BACKER DIRKS, KWANT and KLAASSEN, 1957). The examination was restricted to

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molars and premolars. The palatine pits of the incisors were not evaluated. In the upper molars two fissures were estimated separately (mesio-occlusal and disto-occluso-palatine) while in the lower molars the occlusal fissure and the buccal pit were diagnosed separately. In the premolars the fissures were diagnosed as one unit.

The fissures were cleaned with a new sharp explorer (Maillefer No. 6) and dried with compressed air. The diagnosis was made with the aid of a small mouthlight of high intensity. Incident and transmitted light were used. Caries was estimated in four different grades (I, II, III, IV) but these grades cannot be compared with those for the approximal caries because of the differences in the methods used. Caries I signifies a minute black line at the bottom of the fissure; in caries II there is in addition a white zone along the margins of the fissure (dark in transmitted light); caries III denotes the smallest perceptible break in the continuity of the enamel (cavity) with or without undermined margins. Caries IV is a large cavity more than 3 mm wide. Caries grade III is from a practical point of view comparable to approximal caries II lesions in that both need treatment.

The standardization of a clinical examination is far more difficult than that of a radiographic examination in which the material remains available for checking purposes. By defining the caries stages as exactly as possible and by having always at hand a set of extracted molars showing examples of each diagnosis, an attempt was made to keep the assessment as constant as possible. Because each child was always examined by two dentists and the examinations were alternated weekly between Tiel and Culemborg, it is fairly certain that the examinations in Tiel were made in the same way as those in Culemborg. For all calculations the mean result of the two dentists were used. To what extent the diagnostic criteria were consistent from year to year is unfortunately impossible to determine. The measures described above were used to provide as many safeguards as possible.

For the groups examined, each composed of 100 children, the standard deviation of the estimation was approximately 0.5 per cent.

The examination of the free smooth surfaces was also made after careful cleaning and drying of these surfaces. When necessary an initial cleaning was made with cotton wool or with a tooth brush; in the final cleaning the surface was scraped with the side of the explorer. The examination—likewise by two dentists independently—was restricted to the buccal and labial surfaces. Two stages of caries were distinguished i.e. "caries white" (c.w.) if the surface showed a white chalky opaque lesion and "cariou cavity" (c.c.) if there was a break in the continuity of the enamel perceptible with an explorer.

The time spent on the clinical examination depended upon the number of teeth present in the mouth. The mean examination time spent by each dentist for the groups of 7 years of age was 4.5 min and for the groups of 15 years of age 9.5 min. All the examinations were carried out at the schools. Portable equipment was used.

STUDY GROUPS

The primary purpose of this study pertained to the effect of water fluoridation on approximal caries. This determined the selection of the study groups.

If the water fluoridation is to have practical significance, it must be shown not only that it results in an important percentual reduction of the caries prevalence but also that this reduction, if expressed in numbers of lesions, is of significant practical value. The age group of 11 is the first to show sufficient approximal caries to make this possible. Because of this, the basic material of the investigation was formed by groups of children of 11-15 years old in which the approximal caries was evaluated.

In order to investigate the effect of fluoridation on the other types of caries, a clinical examination was made of some selected age groups.

On the basis of the available data concerning the frequency distribution of caries in the children and the resulting standard deviations of the sample, the standard deviation of the observation and the expected size of the caries preventive effect of the fluoridation, the necessary size of each age class was calculated at 100 children.

Each of the age groups examined contained an equal number of girls and boys, chosen at random from the schools except that allowance was made for the kind of school. From each kind of school (public school, Roman Catholic, Protestant, etc.)

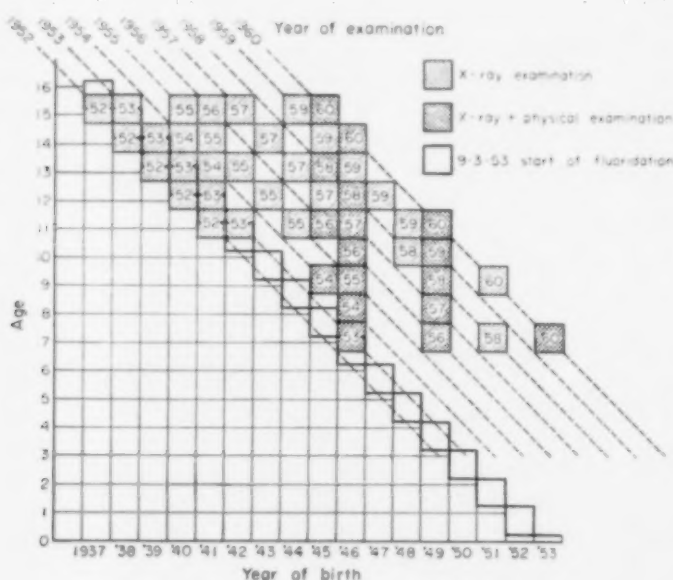


FIG. 1. Age classes studied in the yearly investigations.

a sample was taken in proportion to the number of children attending the school. Only children who had been born in Tiel or Culemborg, and had lived there ever since (except for holidays) and had used the piped water supply, were included in the study.

Fig. 1 gives a survey of the age groups examined with the methods described. As the examination was made always in the last 3 months of the year, and the children grouped by their year of birth, the mean age of the children was 10½, 11½, 12½, etc.

Those groups will be called the 11, 12, 13, etc. year age class. The mean age of the children at the beginning of fluoridation can also be read from Fig. 1.

RESULTS

Approximal caries

First of all the comparability of the children in Tiel and Culemborg as to caries prevalence must be established. In Fig. 2 and Table 1 the average number of approximal lesions per child in 1952 is presented for the group of 11-15 year old children.

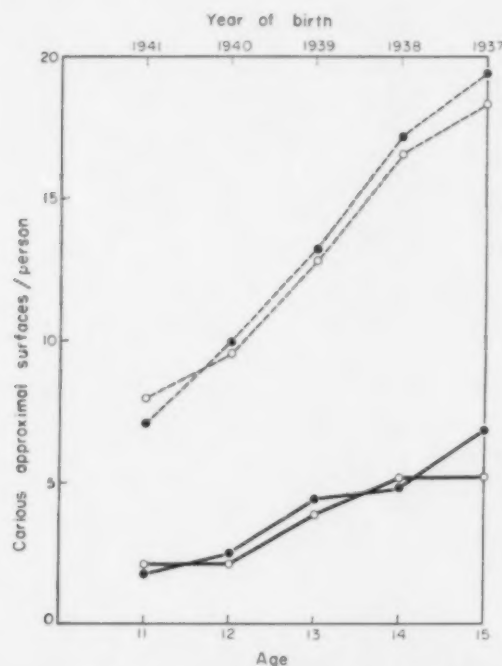


FIG. 2. Average number of carious approximal surfaces per child (broken lines) and the average number of approximal surfaces with caries of the dentine (solid lines), 1952 investigation. ● Culemborg; ○ Tiel.

TABLE 1. AVERAGE NUMBER OF CARIOUS APPROXIMAL SURFACES PER CHILD (I-V) AND AVERAGE NUMBER OF APPROXIMAL SURFACES WITH CARIES OF THE DENTINE PER CHILD (II-V), 1952 INVESTIGATION

None of the differences are significant at the $P=0.01$ level

Age	11		12		13		14		15	
Caries grade	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V
Culemborg	7.1	1.9	9.9	2.4	13.2	4.5	17.2	5.0	19.5	6.5
Tiel	8.2	2.1	9.7	2.4	12.9	3.9	16.7	5.4	18.3	5.3

The graph clearly shows that there were no significant differences between Tiel and Culemborg present at the beginning of the water fluoridation. The minor differences appear to derive from the random composition of the groups. The distributions of the caries lesions over the various tooth surfaces also did not show a significant difference between the two towns. Fig. 3 illustrates this distribution for one age group.

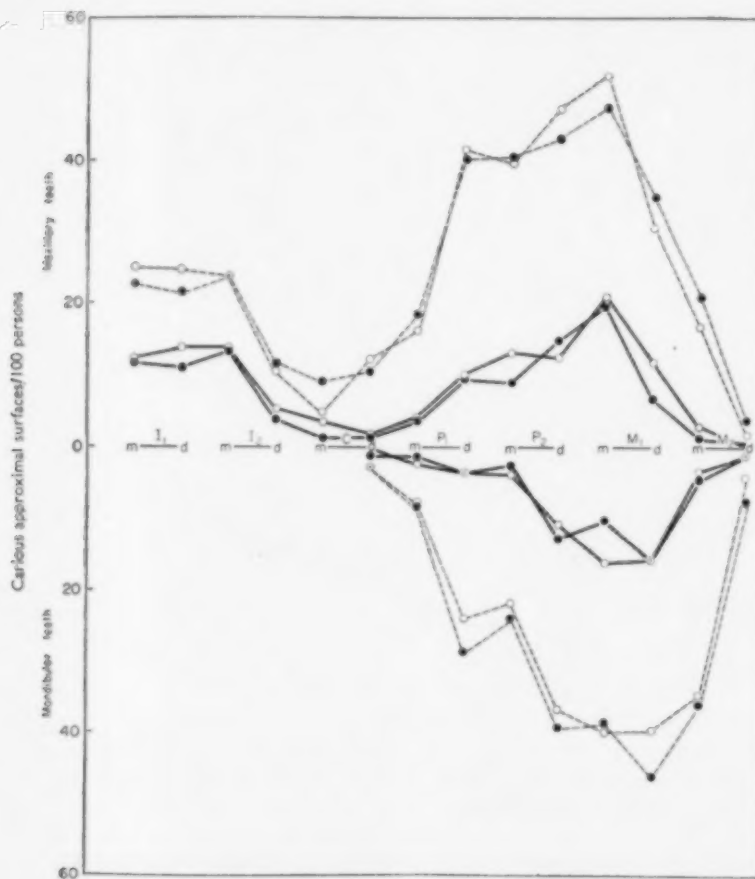


FIG. 3. Number of carious approximal surfaces (broken lines) and of surfaces with caries of the dentine (solid lines) per 100 children of 13 years of age. For each surface the mean number of carious lesions is given for the corresponding surfaces of the right and left mouth half. 1952 investigation. I=incisor; C=canine; P=premolar; M=molar; m=mesial surface; d=distal surface; ●=Culemborg; ○=Tiel.

The effect of fluoridation can be evaluated in various ways:

- (a) Comparison of the caries prevalence in Tiel and Culemborg in the same age groups before and after fluoridation (horizontally in Fig. 1);

- (b) Comparison of the caries prevalence in Tiel and Culemborg in the same year (diagonally in Fig. 1);
- (c) Comparison of the caries increment in Tiel and Culemborg, in the same children (vertically in Fig. 1).

Numbers of approximal carious lesions in Tiel and Culemborg of the age groups 11-15 years

In 1952 and 1953 and every second year thereafter the entire group of 11-15 year old children was examined (see Fig. 1).

In Table 2 and Fig. 4 the average number of approximal lesions (I-V) and the average number of dentinal lesions (II-V) are given for the various investigation years. These averages are obtained from the averages of the component age groups.

TABLE 2. THE AVERAGE NUMBER OF APPROXIMAL LESIONS FOR THE AGE GROUPS OF 11 UP TO AND INCLUDING 15 YEARS; 1952 INVESTIGATION UP TO AND INCLUDING 1959 INVESTIGATION

Investigation year	1952		1953		1955		1957		1959	
Caries grade	I-V*	II-V†	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V
Culemborg	13.4	4.1	12.2	3.6	13.8	4.2	13.5	5.0	13.8	4.8
Tiel	13.1	3.8	12.2	3.7	12.7	4.1	10.7	3.5	10.8	3.1
Percentage difference	—	—	—	—	—	—	21	30	22	35

* I-V=all approximal lesions. † II-V only approximal dentinal lesions.

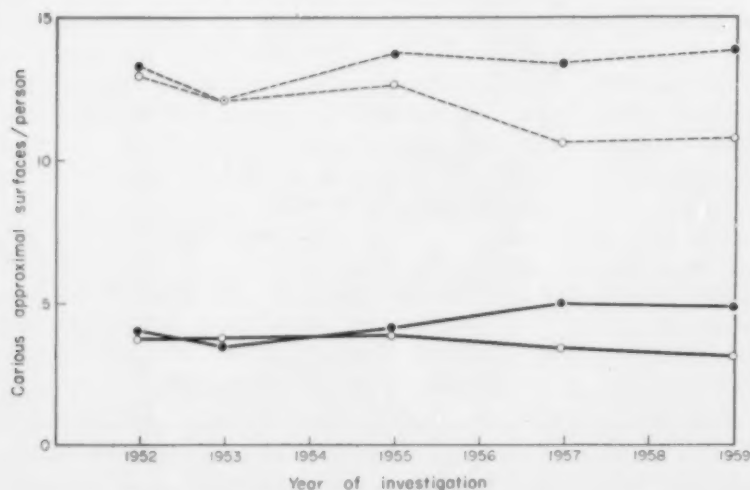


FIG. 4. Average number of carious approximal surfaces (broken lines) and of surfaces with caries of the dentine (solid lines). 11-15 years age group. ● Culemborg; ○ Tiel.

The average of each component age group is always the average number of lesions of the boys' and girls' mean number of lesions.

For the study years of 1952 and 1953 there is no difference between Tiel and Culemborg, in 1955 there is a limited difference and in 1957 and 1959 a clear-cut difference which favours Tiel.

In 1953 the values for the total number of lesions for both Tiel and Culemborg were strikingly low. In analysing the 1953 results it appears that this low average is due to the low caries numbers of the 14 and 15 year old children. It turned out that the radiographs of these age classes were slightly underdeveloped, with the result that especially many of the slightest carious lesions (minute enamel caries) were not diagnosed. These radiographs were developed in the very cold month of February 1954, by a temporary assistant, who did not check adequately the temperature of the development bath. As a consequence the average of 1953 cannot be compared with the averages of the other years. The values for Tiel and Culemborg are, however, mutually comparable since the radiographs were mixed before development. The susceptibility of this method is clearly demonstrated and emphasizes the need for a rigorous standardization of all the methods used in the investigation.

In Culemborg, except for the figure of 1953, the total number of lesions (I-V) is very constant. For Tiel however the numbers for the year 1957 and 1959 are about 20 per cent lower than the numbers for Culemborg and also lower than the numbers for Tiel in 1952.

By contrast the number of dentinal lesions (II-V) in Culemborg shows an important increase while on the other hand Tiel shows a decrease in dentinal lesions of the same magnitude.

The constant increase in caries prevalence is a common observation in Holland as well as in other countries. It seems therefore more logical to compare the caries numbers in the same year of investigation. The basis of the study is the comparability of the two towns. There is no reason to suppose that Tiel (unlike Culemborg) would not have shown, without fluoridation, an increased caries prevalence. The simultaneous development and the blind examination reduces to a minimum the chance for differences in quality of the radiographs and in caries criteria between Tiel and Culemborg within the same year of investigation.

The comparison of averages of such relatively broad age groups (11-15 years) are in general hardly permissible as the oldest age class will have too dominant an effect on the averages. Especially for Tiel this average gives a distorted picture as the youngest age groups (11-12 years) which show the greatest caries reduction hardly affect the mean value of the whole group, whereas a relatively small caries reduction in the oldest age group has an important effect on the mean value.

Number of approximal lesions in Tiel and Culemborg in the same investigation year

In Fig. 2 it has been shown already that there were no significant differences in caries prevalence in Tiel and Culemborg at the beginning of the fluoridation. In 1955 almost all caries figures were somewhat lower in Tiel than in Culemborg. After 4½ years of fluoridation (1957) a distinct difference in the number of carious lesions was found; practically all differences were significant at the 1 per cent level.

The caries figures for 1959 (6½ years after the beginning of fluoridation) are shown in Table 3 and Fig. 5. The small vertical lines in this figure are twice the standard deviation of each average, put one time above and one time below the average.

With the exception of the caries grades I-V of the 14 year old group and of the caries grades II-V of the 15 year old group, all *P* values for the differences are smaller than 1 per cent.

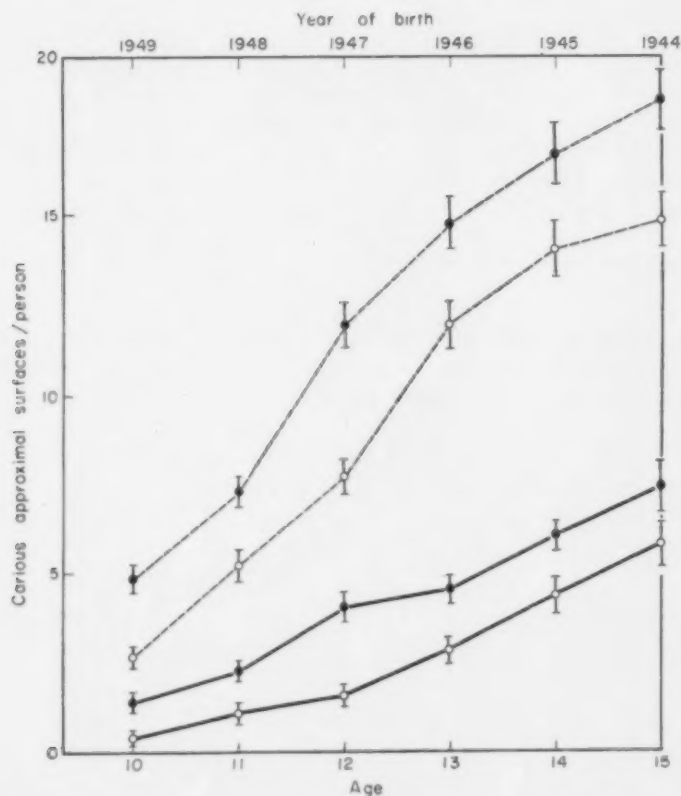


FIG. 5. Average number of carious approximal surfaces per child (broken lines) and the average number of approximal surfaces with caries of the dentine (solid lines), 1959 investigation. The small vertical lines give the standard deviation of the mean (σ_m) above and below the observed averages. ● Culemborg; ○ Tiel.

It can be observed that the differences—when expressed as percentages less caries—are larger in the younger age groups and smaller in the older age classes. This is well in accord with the hypothesis that the water fluoridation is the cause of these differences. The younger the age of the children at the start of fluoridation, the more important the effect should be.

TABLE 3. AVERAGE NUMBER OF CARIOUS APPROXIMAL SURFACES PER CHILD (I-V) AND AVERAGE NUMBER OF APPROXIMAL SURFACES WITH INVOLVEMENT OF THE DENTINE PER CHILD (II-V), 1959 INVESTIGATION

The averages are rounded off at one decimal fraction, the percentages at whole numbers. C.R. (critical ratio)=number of times the difference between Culemborg and Tiel is larger than the standard error of the difference

Age	10		11		12		13		14		15	
Year of birth	1949		1948		1947		1946		1945		1944	
Age at start of fluoridation	3½		4½		5½		6½		7½		8½	
Caries grade	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V	I-V	II-V
Culemborg (<0.1 mg F/l)	4.9	1.5	7.3	2.3	11.9	4.0	14.7	4.5	16.7	6.0	18.2	7.4
Tiel (1.1 mg F/l)	2.7	0.5	5.3	1.1	7.7	1.6	12.0	2.8	14.0	4.3	14.8	5.8
% less caries lesions	45	67	27	50	35	60	18	38	16	28	19	22
C.R. of the difference	6.84	5.10	3.29	3.86	5.04	6.59	2.77	3.75	2.42*	2.52	3.22	2.01*

* Differences not significant at the $P=0.01$ level.

In a binominal frequency distribution the average with its standard deviation is a good description of the observed values. However, if the frequency distribution is very skew, like the frequency distribution of the carious lesions in the children, the

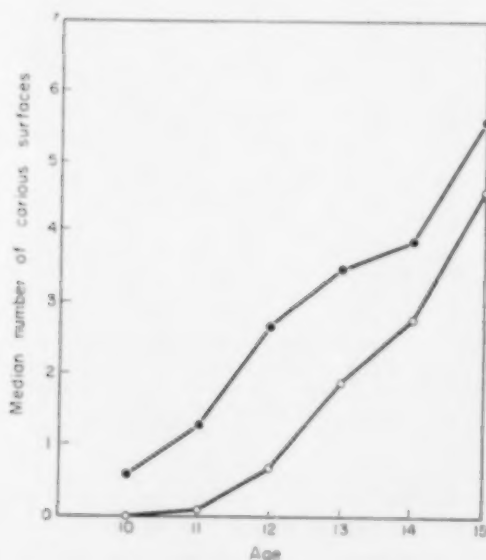


FIG. 6. Median number of approximal surfaces with lesions of the dentine per child, 1959 investigation. ● Culemborg; ○ Tiel.

TABLE 4. MEDIAN NUMBER OF SURFACES WITH DENTINAL LESIONS PER CHILD

Age	10	11	12	13	14	15
Culemborg	0.6	1.3	2.7	3.5	3.9	5.6
Tiel	0.0	0.1	0.7	1.9	2.8	4.6

average is less appropriate to describe the caries prevalence. In order to give a better insight into the caries prevalence Fig. 6 and Table 4 show the median values of the caries degrees II-V (dentinal lesions) for the same groups. The differences in the median values, especially for the younger age groups, are larger than the differences in the mean values.

Caries increment in Tiel as compared to Culemborg

As a number of the groups studied already had caries at the beginning of fluoridation, caries increment will give a better picture of the fluoride effect than caries prevalence (=lifetime caries experience).

In Table 5 are presented the number of approximal dentinal lesions at various ages for the four groups of children born in 1944, 1945, 1946 and 1949. As these caries numbers are calculated only for those children who took part in the subsequent examinations, the figures differ from those of Table 3. All differences in caries increment are larger than the differences in caries prevalence (Table 3).

TABLE 5. APPROXIMAL CARIOUS LESIONS WITH INVOLVEMENT OF THE DENTINE AT DIFFERENT AGES
Average number per child

Age at examination	Year of birth							
	1944		1945		1946		1949	
	C	T	C	T	C	T	C	T
7							0.0	0.1
8							0.5	0.1
9			0.6	0.9	0.7	0.5	0.9	0.3
10							1.5	0.5
11	2.1	2.4	2.3	1.8	2.5	1.6		
12			4.1	2.8				
13	4.9	4.5	5.4	3.6	4.6	2.8		
14			6.2	4.4				
15	7.5	6.0						
Total caries increment	5.4	3.6	5.6	3.5	3.9	2.3	1.5	0.5
% difference	33		37		41		67	

C=Culemborg; T=Tiel.

For the same children the number of approximal lesions and the number of dentinal lesions are shown graphically in Fig. 7 for the various years of investigation. For Culemborg the curves of the various groups are close to each other. The slope of the curves, i.e. the caries increment, is very similar: all groups from Culemborg belong to the same population ("universe"). For Tiel this is apparently not true and proves the dissimilarity of the four groups. The slope of the curves decreases for each year later the children are born. Each group had a different age at the beginning of fluoridation, and is therefore not protected in the same way.

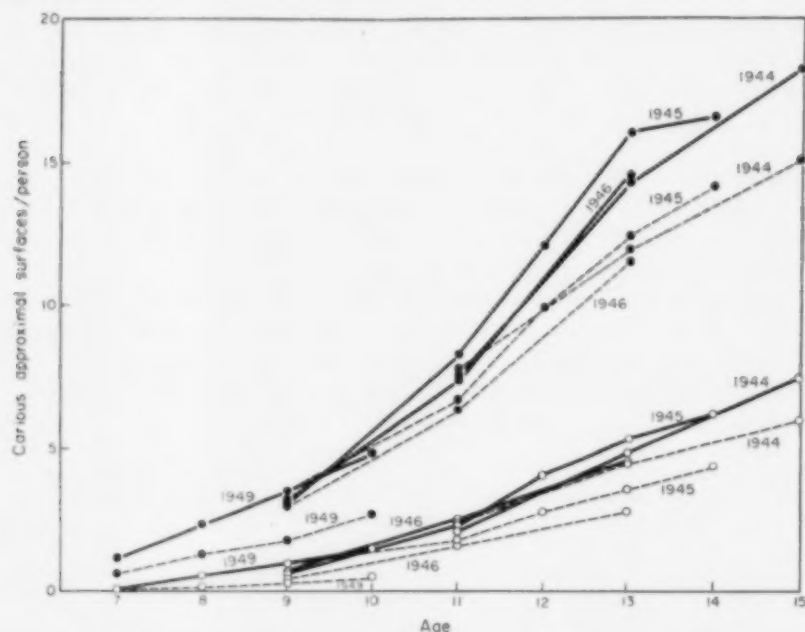


FIG. 7. Mean number of approximal carious surfaces per child at different ages. Longitudinal study of groups of children from Culemborg (solid lines) and from Tiel (broken lines). Year of birth respectively 1949, 1946, 1945 and 1944. ● all carious surfaces; ○ surfaces with caries of dentine.

For all age periods the slope of the curves pertaining to Tiel is less steep than those of Culemborg.

The increment in approximal lesions of the dentine over a 4 year period was for the oldest age group born in 1944 and with a mean age of $8\frac{1}{2}$ years at the beginning of fluoridation 33 per cent smaller in Tiel than in Culemborg.

Pit and fissure caries

In the 1959 investigation only the children of 10 years old (born in 1949) were examined for fissure caries. Therefore the caries figures of the three age groups studied in the 1958 investigation will be shown. In this year the children born in 1945, 1946 and 1949 (9, 12 and 13 years of age) were examined clinically (see Fig. 1).

Whereas caries grade III is clinically comparable to caries grade II of the approximal lesions and caries grade II resembles caries grade I of the approximal lesions, caries figures will be presented in two categories: caries grades III, IV and fillings (denoted as caries III-V) and caries grades II, III, IV and fillings (caries II-V). (In the calculation for pit and fissure caries grade I is evaluated as sound). Extractions are calculated as caries grade IV, except orthodontic extractions of premolars.

In Table 6 and Fig. 8 the data of the various age classes are shown.

TABLE 6. AVERAGE NUMBER OF PIT AND FISSURE LESIONS PER CHILD, 1958 INVESTIGATION

Age Year of birth Caries grade	9 1949		12 1946		13 1945	
	II-V	III-V*	II-V	III-V	II-V	III-V
Culemborg	6.6	5.7	12.3	10.0	14.2	11.4
Tiel	5.4	4.1	10.0	7.8	13.0	9.6
% less carious lesions	18	28	18	22	8	16
C.R. of the difference	4.6	5.7	4.3	4.6	2.2†	3.5

Extractions are calculated as caries grade IV.

* III-V=carious cavities only.

† Difference not significant at the $P=0.01$ level.

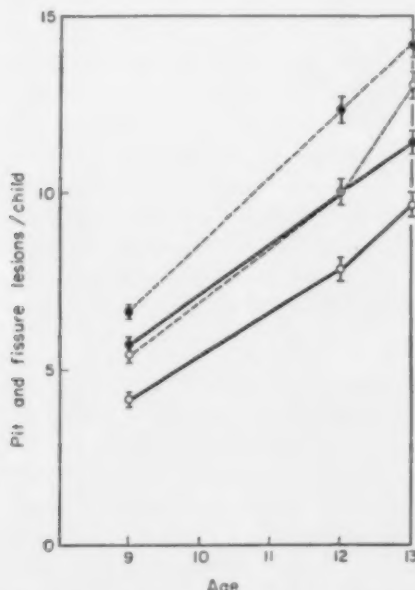


FIG. 8. Average number of pit and fissure lesions per child (broken lines) and average number of pits and fissures with cavitation (solid lines). The small vertical lines give the standard deviation (σ_m) above and below the observed averages.

● Culemborg; ○ Tiel.

The percentage differences in carious lesions of the fissures is much smaller than the differences in lesions of the approximal surfaces. Even in the youngest children, who were $3\frac{1}{4}$ – $4\frac{1}{4}$ years old at the beginning of fluoridation, the reduction in the number of carious cavities is only 28 per cent. For the approximal surfaces a caries reduction of 67 per cent was calculated (compare Table 3). Either pits and fissures are affected less by fluoride than approximal surfaces or fluoride must be present in a much earlier phase of tooth development, than for approximal surfaces, to have a comparable caries-preventive effect (BACKER DIRKS, HOUWINK and KWANT, 1961).

Caries of the free smooth surfaces

The carious lesions of the free smooth surfaces were diagnosed during the clinical examination. For the same reason as mentioned in respect of lesions of fissures the results of the 1958 investigation will be shown.

TABLE 7. NUMBER OF CARIOUS LESIONS OF THE SMOOTH BUCCAL AND LABIAL SURFACES PER 100 CHILDREN

Age Year of birth Caries grade	9 1949		12 1946		13 1945	
	c.w.*	c.c†	c.w.	c.c.	c.w.	c.c.
Culemborg	175	45	383	86	472	96
Tiel	98	14	269	25	310	43
% less carious lesions	44	69	29	71	34	55

* c.w. = white chalky lesions, without macroscopic loss of substance.

† c.c. = carious lesions with cavitation.

In Table 7 the numbers of buccal or labial lesions of smooth surfaces are presented for three age groups from Culemborg and Tiel. The differences between Tiel and Culemborg in the number of cavities (c.c.) are particularly large in comparison with the approximal surfaces and the occlusal surfaces. Even those surfaces which had already erupted at the beginning of fluoridation (first molars and incisors of the 1945 age class) did show an important caries reduction (40 per cent).

DISCUSSION

The differences in the methods of examination and in the definition of the caries criteria make it difficult to compare the results of this study with the results of fluoridation in the U.S.A. With this in mind it may be concluded that water fluoridation in the Netherlands has a favourable caries inhibiting effect which is fairly comparable to the results of the American studies after the same period. The Dutch governmental committee on "Dental caries and fluorides" recommended, upon the basis of these studies, water fluoridation in the Netherlands (HEALTH COUNCIL, 1960).

The data shown give the effect of fluoridation after $6\frac{1}{2}$ years in children of 10–15 years of age. As the mean age of these children was $3\frac{1}{4}$ – $8\frac{1}{4}$ years at the start of fluorida-

tion the great majority of the teeth studied was already calcified or even erupted at this time. In view of the results of the American studies it may be concluded that the caries inhibiting effect of water fluoridation will become still larger when children born in later years are included in the study.

The observation that the fluoride effect is largest for lesions on free smooth surfaces, slightly less for approximal surfaces and smallest for occlusal (pits and fissures) caries is of definite importance. From the theoretical point of view this suggests that the accessibility of the various surfaces to fluoride ions both pre- and posteruptively, is a decisive factor in the fluoride effect (see also BACKER DIRKS *et al.*, 1961). Before eruption the mesial, distal and buccal surfaces will be accessible to the fluoride-containing tissue fluids. However, for the deep occlusal fissures the accessibility will be far less. When after eruption the mesial and distal surfaces become contact surfaces the situation is no longer favourable for the adsorption of fluoride ions from saliva. The diffusion route through plaque material will be much longer in the molar region than in the anterior region (WINKLER and BACKER DIRKS, 1958). The smooth buccal surface remains a freely accessible surface. If this supposition is right, the occlusal fissure will accumulate much less fluoride in the outer enamel than the other surfaces (ISAAC *et al.*, 1958).

This difference in effect is also of practical importance. The fact that the caries site which gives little difficulty in dental treatment is the least influenced by fluoride is a fortunate coincidence.

It is obvious from Tables 3, 6 and 7, that the percentual reduction of caries due to fluoridation is more marked for dentinal lesions only, than for all lesions. This shows that fluoride also inhibits the increase in size of small enamel lesions. It is possible that a rehardening of the initial lesion is promoted by the presence of fluoride, as suggested by the experiments of KOULOURLIDES, CURTO and PIGMAN (1961), or that a selective absorption of fluoride ions takes place in the initial enamel lesion.

The differences in caries inhibition, if assessed from the mean caries numbers or from the median caries numbers, lead to the conclusion that the children with the highest caries susceptibility are least protected by fluorides (Figs. 5 and 6).

Acknowledgements—We wish to express our sincere gratitude to Professor Dr. K. C. WINKLER for his advice and help during the study. The execution of important tasks, for example the examination of radiographs, by Misses L. BAUER, J. BROUWER and J. VAN WACHEM and Mr. C. M. VELTEMA is acknowledged with great appreciation.

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DEVELOPMENTAL AND POST-DEVELOPMENTAL INFLUENCES ON INCIDENCE OF EXPERIMENTAL DENTAL CARIES RESULTING FROM DIETARY SUPPLEMENTATION BY VARIOUS ELEMENTS

J. H. SHAW and D. GRIFFITHS

Harvard School of Dental Medicine, Boston 15, Massachusetts, U.S.A.

Abstract—Caries-susceptible rats of the Harvard strain were used to evaluate developmental and post-developmental influences of dietary supplements of several mineral salts on experimental dental caries. When various levels of sodium chloride, barium chloride, vanadium pentoxide, vanadyl sulphate and ammonium paramolybdate were fed to Harvard caries-susceptible rats, these mineral salts had no consistent, statistically significant influence on the dental caries incidence. Sodium borate and strontium carbonate supplements post-developmentally caused modest but rather inconsistent reductions in the dental caries incidence. Lithium carbonate supplements fed post-developmentally caused consistent reductions in the incidence of dental caries that were significant at the 5 per cent level, while ammonium molybdate supplements under the same experimental circumstances caused reductions that were statistically significant at the 1 per cent level.

The various levels of barium chloride, sodium borate, and ammonium molybdate that were provided during pregnancy and lactation in the effort to influence the development of the molars of the offspring had no influence on the dental caries susceptibility of the offspring. Developmental supplements of vanadium pentoxide and vanadyl sulphate resulted in inconclusive findings due to poor reproduction in these experiments, but there seemed to be little evidence that the dental caries susceptibility of the offspring had been altered by either vanadium compound. Developmental supplements of sodium chloride, lithium carbonate, strontium carbonate and ammonium paramolybdate resulted in significant increases in the dental caries susceptibility of the offspring at levels varying from 5 per cent to 1 per cent.

Many meticulous experiments need to be conducted before there will be detailed understanding of the role of the minor elements in the development and maintenance of the teeth.

INTRODUCTION

SUPPLEMENTS of a wide variety and combination of anions and cations to cariogenic regimens have been tested for their ability to influence the dental caries activity in rodents (HEIN, 1955). The influence of most supplements was tested after the crowns of the molar teeth had been calcified fully and after most molar teeth had erupted into the oral cavity. Therefore most of these results represent evaluations of post-developmental influences of the test agents upon the oral milieu and upon tooth surfaces rather than evaluations of the developmental influence of the elements tested upon the components of the teeth. The present series of experiments has been undertaken with caries-susceptible rats as an effort to evaluate not only the post-developmental but also the developmental influence of dietary supplements of several individual mineral elements as well as crude mixtures containing a variety of elements.

EXPERIMENTAL DESIGN AND RESULTS

White rats from the Harvard caries-susceptible colony were carefully selected at weaning to ensure uniformity of caries-susceptibility within each experiment to be reported. Since these experiments were conducted over a period of 4 years, the caries-susceptibility from one experiment to another varies somewhat as various segments of the strain were used and as the strain had been improved by selection prior to the later experiments. The breeding stock in this colony has been maintained for several generations without interruption on diet 700+15 per cent Cellu flour (RESNICK, WILLETT and SHAW, 1958), which recently has been renumbered diet 2700 (SHAW and GRIFFITHS, 1960).

Litters were distributed uniformly among the control and experimental groups. Throughout all experiments the control ration was 2700 or a minor modification thereof. The experimental diets consisted of diet 2700 to which was added an appropriate amount of one of the mineral supplements under investigation. The highest level of any supplement was selected near the lower range of toxicity in order to bring the maximum influence to bear that was consistent with the purpose of the particular experiment. The amount of each mineral supplement was calculated and is expressed throughout the manuscript in terms of percentage or parts per million (p.p.m.) of a 100 g aliquot of the sucrose, casein, salt mixture and corn oil in the diet. The 4 per cent whole liver substance and the 15 per cent Cellu flour were excluded from these calculations, since they were considered to be subsidiary supplements and not absolutely vital to the maintenance of the rats.

All rats were maintained in screen-bottomed cages except during the reproductive periods, when the males and females were housed in cages with soft pine shavings as bedding. Tap water and the diets were provided to all animals *ad libitum* throughout the experiment. Weekly body weights were recorded.

Each experiment was composed of two parts: the parental generation and the filial generation. The members of the parental generation had been selected from the colony to serve either one of two functions: breeders for the production of the filial generation or to test the post-eruptive influence of the experimental diets on the caries incidence in comparison to that of the rats fed the control ration. At an appropriate time when gross carious lesions could be detected and readily classified with respect to size during *in vivo* observations, a small number of closely matched, littermate males and females were selected for mating from each control group and from most experimental groups. Their dietary regimens were continued throughout the reproductive cycle in order to provide the opportunity to influence the development of teeth in the filial generation.

Breeders were not selected from every experimental group. In cases where two levels of a mineral supplement were being studied and both proved to be relatively innocuous, breeders were selected only from the higher supplementation level. In other cases where one or more levels of a supplement proved to be too toxic in the maternal generation, no breeders were selected.

The rats not selected for breeding were sacrificed at an appropriate time for evaluations of the incidence of experimental dental caries as influenced by purely

post-developmental routes. The time of sacrifice was determined independently in each experiment on the basis of the caries-susceptibility of the subjects. As far as the breeders were concerned, their caries evaluations were excluded from these averages because their periods on the dietary regimens were much longer, indeed too long to be meaningful in the post-developmental evaluation of the influence of any supplement upon caries incidence.

The rate of growth and the ability of the animals to reproduce and rear offspring during a prolonged period on the experimental diets were observed. Since the progeny were born and reared during the time the parents were being sustained by an experimental diet, the caries incidences of these offspring were compared with the progeny of the control females to determine the influence of these dietary supplements during tooth development on the inherent caries-susceptibility.

In all experiments, each litter in the filial generation which was born and reared by a female on an experimental diet was divided into Subgroups A and B at weaning. The offspring in Subgroup A of each experimental group consistently were fed control diet 2700, while those in Subgroup B were continued on the supplemented diet of their parents. The rats in Subgroup A were used to determine the influence on dental caries incidence of the dietary supplements when fed only during pregnancy and lactation. The offspring in Subgroup B provided an opportunity to test whether the supplements had any different effect when fed throughout the entire experimental duration, i.e. not only during development, but post-developmentally as well.

All offspring from the females in the control group of any experiment were maintained on diet 2700 in most of the experimental trials; however, in some experiments the offspring of the control females were divided into two Subgroups, A and B, to provide further post-developmental tests of the dietary supplements. In these cases, the offspring in Subgroup B were continued on ration 2700, the diet provided to their parents, while the offspring in Subgroup A received a mineral supplement as a further test of the possible post-developmental influence in a second generation. Thus in all Subgroups B, experimental or control, the diet fed was the same as that of the parents, whereas in all Subgroups A, experimental or control, there was a change of diet for the offspring. Hence in all tables the diet listed in the second columns represent the diet of that particular generation, but in addition the diet of the maternal generation for that breeding combination can be determined by noting the diet recorded in column 2 for Subgroup B.

In order to reduce confusion between experiments, the groups have been given numerical designations beginning in the first experiment with control Group 1 and continuing consecutively to 47 for the last experimental group in the sixteenth experiment. Likewise, post-developmental data obtained from the maternal generation are presented in Tables 2, 4 and 7 and developmental data from the filial generation in Tables 1, 3, 5, 6 and 8.

The experimental periods varied from 95 to 145 days in the various experimental trials as were determined for each experiment on the basis of the time when gross carious lesions could be observed *in vivo* in the control groups to be at a desirable level for evaluation *in vitro*. For all experiments, the rats were sacrificed by an

overdose of ether and then decapitated; the heads were preserved in 95% alcohol. The molars were evaluated for carious lesions by the procedure of SHAW *et al.* (1944).

1. *Crude sea salt and sodium chloride supplements*

In the first experiment, the developmental and post-developmental influences of supplements of varying levels of sodium chloride and of three crude salt preparations were evaluated. The first group of rats served as controls and was fed a diet designated as 2702+1.5 per cent sodium chloride. This diet was identical in composition to control diet 2700 and differed only in the manner of preparation to suit the experimental design. The reagent grade salt mixture in diet 2702 was identical to that in diet 2700 except for the total omission of sodium chloride. Without a sodium chloride replacement, diet 2702 would be sodium and chloride deficient. When diet 2702 was supplemented with 1.5 per cent sodium chloride, it had the same quantity and distribution of minerals as diet 2700.

The rats in Group 2 were provided diet 2702 with a 2 per cent supplement of a local sea salt (Admiral Trace Element Salt, Sea Minerals, Inc., Houston, Texas) prepared commercially in this country in a fine, white granular form that could be readily incorporated into the diet. This supplement provides approximately the same amount of sodium and chloride as a 1.5 per cent sodium chloride supplement but in addition provides the wide distribution of other elements present in sea water. The rats in Groups 3-6 received supplements of 3.0 per cent sodium chloride, 4.0 per cent of the local sea salt, 6.0 per cent sodium chloride and 8.0 per cent of the local sea salt, respectively.

The rats in Group 7 received a supplement of 4.0 per cent crude Indian sea salt imported from India. This material was composed of a mixture of irregularly shaped chunks of various sizes ranging from light tan to light grey in colour. Presumably this material had the form and composition typical of its preparation in settling basins along the ocean front for local consumption as a condiment. When finely ground in preparation for use in the diet, this material was slightly hygroscopic and caked easily. The rats in Group 8 received a supplement of 4.0 per cent of Sambhar Lake salt. This material was also imported from India where it was mined in the north for local use as a condiment. This material was made up of large crystalline masses of a pale amethyst hue. When finely ground, this preparation was slightly hygroscopic and light pink. Both the Indian sea salt and the Sambhar Lake salt contained from 65 to 75 per cent sodium chloride and a wide variety of other elements. Their fluoride concentrations varied from 35 to 55 p.p.m.

The rats sacrificed in the maternal generation to study the post-developmental effects of these supplements were on experiment for 115 days, while the offspring were sacrificed after 103 days on experiment.

The data for the post-developmental influence of these supplements upon the rates of growth and upon the dental caries incidence of the fourteen or fifteen rats in each group of the maternal generation are not presented in detail because they were almost entirely negative. The average weight increases for the male rats in these eight groups were relatively similar; however, there was a slight tendency for

the higher levels of supplementation to depress the rate of growth. This depression in growth rate was particularly evident for the rats fed diet 2702+8 per cent of the local sea salt. The average weight increases for the female rats tended to be a little more depressed by the higher levels of supplementation than occurred among the males.

Some minor variations in dental caries incidence occurred from group to group but only one difference between an experimental group and the control group was statistically significant at the 5 per cent level. This difference occurred for the number of carious areas in the comparison between the controls in Group 1 and the experimentals in Group 2 where 2 per cent of the local sea salt was used in place of 1.5 per cent sodium chloride. Since no significant difference was observed for the number of carious molars nor for the extent of caries scores for the same two groups of rats, it seems unlikely that any importance should be attached to this difference.

TABLE 1. THE DEVELOPMENTAL INFLUENCE OF VARIOUS AMOUNTS AND VARIOUS SOURCES OF SODIUM CHLORIDE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
1A	2700	4	3	259	172	5.1	0.9	—	6.9	1.7	—	21.6+	6.7+	—
1B	2702+1.5% NaCl	5	3	234	162	4.8	0.6	0.3	6.6	1.3	0.1	18.1+	3.5+	0.5
2A	2700	8	10	285	163	6.5	0.4	1.4	10.1	1.0	1.6	29.7+	3.1+	1.1
2B	2702+2.0% Trace element sea salt	10	10	286	158	6.8	0.4	1.7	10.7	1.0	1.9	32.4+	4.0+	1.4
3A	2700	13	7	271	160	6.3	0.5	1.2	11.0	1.3	1.9	35.4+	4.6+	1.7
3B	2702+3.0% NaCl	11	9	239	151	6.6	0.5	1.5	12.3	1.0	2.7	40.2+	3.9+	2.4
4A	2700	12	9	260	151	6.3	0.4	1.2	10.1	0.9	1.7	30.0+	2.9+	1.2
4B	2702+4.0% Trace element sea salt	10	10	219	151	6.1	0.5	1.0	9.7	1.2	1.3	31.0+	4.9+	1.1
5A	2700	6	13	272	149	7.7	0.3	2.7	14.7	0.9	4.1	46.9+	3.1+	3.4
5B	2702+6.0% NaCl	4	14	260	142	7.7	0.4	2.7	14.7	1.0	4.0	46.6+	3.6+	3.3
6A	2700	12	21	267	160	5.5	0.4	0.4	9.7	0.9	1.5	31.0+	3.4+	1.3
6B	2702+8.0% Trace element sea salt	17	15	237	145	6.1	0.3	1.1	10.2	0.8	1.8	31.5+	3.4+	1.3
7A	2700	5	5	229	130	5.6	0.7	0.4	9.0	1.5	0.9	28.3+	4.7+	0.8
7B	2702+4.0% Indian sea salt	3	6	231	168	5.6	0.8	0.4	8.8	2.0	0.7	27.8+	7.1+	0.6
8A	2700	6	14	266	148	7.1	0.5	1.9	13.2	1.4	2.9	44.0+	5.8+	2.5
8B	2702+4.0% Sambhar Lake salt	8	11	251	142	6.7	0.4	1.6	11.1	1.0	1.2	35.6+	4.2+	1.8

* SEM—Standard error of mean.

† CR—Critical ratio. The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. All comparisons are made with the value for Group 1A.

The data for the developmental influence of the same supplements in the filial generation are presented in Table 1. Again growth rates were a little depressed among

the rats on the higher levels of supplementation, with the females more affected than the males. The reproductive ability of the members of the maternal generation was not affected adversely by any level of supplementation.

The offspring of the experimental groups in every case had higher average numbers of carious molars, higher average numbers of carious areas and higher average extents of caries scores than the comparable values for the offspring of the control group. The greatest increases in dental caries incidence were observed in the offspring of experimental Group 5 fed a supplement of 6.0 per cent sodium chloride during pregnancy and lactation. The averages for the number of carious areas and the extent of caries score in this experimental group were significantly greater than the averages for the control group at the 1 per cent level. The difference in the average for the number of carious molars between the experimental and control group approach significance at the 1 per cent level. The next highest increases were observed among the offspring of the groups fed 4.0 per cent Sambhar Lake salt and 3.0 per cent sodium chloride throughout pregnancy and lactation where most differences were significant at the 5 per cent level. The increases for the remaining four experimental groups were not of themselves statistically different from the averages of the controls; however, the fact that all the experimental groups without exception had higher averages than the control rats further contributes to the general impression of the caries-accentuating influences of these supplements during pregnancy and lactation. It is noteworthy that the complex supplements, the local sea salt, Indian sea salt and Sambhar Lake salt, did not differ in their influence from that of reagent grade sodium chloride. Hence we must conclude that the elevated sodium chloride content in the diet for all experimental groups was responsible for the increased caries-susceptibility of the offspring. Furthermore, the wide variety of other elements in the complex supplements was not capable of counteracting the influence of sodium chloride.

From these data obtained in the filial generation primarily to assess the developmental influences of these supplements, it is obvious that the post-developmental feeding of the supplement did not alter the caries activity of the experimental subjects. This generalization applies in all eight groups, where no significant difference existed between Subgroup A on diet 2700 and Subgroup B with a supplement, irrespective of the dietary regimen maintained during pregnancy and lactation. These data in the filial generation coupled with those from the maternal generation clearly demonstrated the lack of a post-developmental influence of any of these supplements.

2. Barium chloride supplements

The second experiment was composed of Groups 9, 10 and 11 and was planned for the evaluation of the post-developmental and developmental influences of barium chloride supplements at levels of 0.5 and 1.0 per cent in diet 2700. The rats in the post-developmental section of the maternal generation were killed after 98 days on experiment, while the offspring in the filial generation were killed after 120 days on experiment.

TABLE 2. THE POST-DEVELOPMENTAL INFLUENCE OF SUPPLEMENTS OF BARIUM CHLORIDE AND SODIUM BORATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
9	2700	13	12	223	115	7.6	0.6	—	13.9	1.6	—	41.5+	5.1+	—
10	2700+0.5% BaCl ₂	14	12	190	123	8.5	0.6	1.1	15.5	1.3	0.8	40.6+	3.5+	0.1
11	2700+1.0% BaCl ₂	10	11	167	112	7.1	0.6	0.7	11.5	1.5	1.1	30.5+	3.8+	1.8
12	2700	13	13	198	108	6.9	0.7	—	11.8	1.6	—	35.3+	5.2+	—
13	2700+0.5% Na ₂ B ₄ O ₇ ·10H ₂ O	16	15	192	98	4.9	0.4	2.5	8.0	0.8	2.1	25.0+	2.8+	1.7
14	2700+1.0% Na ₂ B ₄ O ₇ ·10H ₂ O	13	12	196	104	4.4	0.5	2.9	7.4	0.9	2.4	21.0+	3.0+	2.4
15	2700	3	3	179	93	4.0	1.0	—	6.5	1.5	—	23.2+	6.1+	—
16	2700+2.0% Na ₂ B ₄ O ₇ ·10H ₂ O	3	3	30	54	3.3	1.0	0.5	5.2	1.8	0.6	20.8+	7.2+	0.3

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons for Groups 10 and 11 made with Group 9, for Groups 13 and 14 with Group 12 and for Group 16 with Group 15.

The post-developmental data from this experiment are presented in Table 2. The growth rates of the female rats were not altered by these levels of barium chloride ingestion, whereas the males were definitely penalized. In a pilot experiment, a group of rats fed a supplement of 2.0 per cent barium chloride was unable to survive the experimental period. The 0.5 and 1.0 per cent supplements of barium chloride in Groups 10 and 11 had no statistically significant influence on the incidence of experimental dental caries when provided on a post-developmental basis. The group of twenty-one rats on the 1.0 per cent barium chloride supplement tended to have a lower dental caries incidence than those of either other group, especially with respect to the average extent of caries scores.

The data for the developmental influence of the barium chloride supplements are presented in Table 3. The reproductive ability in the maternal generation was not affected by the barium chloride supplements during pregnancy and lactation. The offspring of the barium-supplemented groups grew approximately as well as those of the control group. The latter observation was true for those rats in Subgroups A fed diet 2700 post-weaning and also for those in Subgroups B fed diet 2700 with barium chloride supplements after weaning. The average dental caries incidences for the offspring in Groups 10A and 10B were slightly but not significantly lower than for the control offspring in Group 9B except for the average number of carious molars in Group 10B, where the difference was barely significant at the 5 per cent level. This reduction apparently was attributable to a post-developmental rather

TABLE 3. THE DEVELOPMENTAL INFLUENCE OF SUPPLEMENTS OF BARIUM CHLORIDE AND SODIUM BORATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
9B	2700	22	20	268	152	6.8	0.4	—	12.1	0.9	—	37.8+	3.5+	—
10A	2700	13	11	282	160	6.2	0.6	0.9	10.4	1.4	1.0	31.4+	5.0+	1.0
10B	2700+0.5% BaCl ₂	11	13	264	157	5.2	0.7	2.0	10.2	1.5	1.1	31.9+	4.8+	1.0
11A	2700	7	7	246	189	7.3	0.7	0.6	13.7	1.0	1.2	44.3+	6.7+	0.9
11B	2700+1.0% BaCl ₂	7	8	262	165	5.0	0.6	2.5	7.0	1.2	3.4	20.7+	3.8+	3.4
12B	2700	4	7	281	148	5.1	0.8	—	8.6	1.8	—	25.3+	5.0+	—
13A	2700	5	5	265	157	6.6	0.8	1.3	12.3	2.5	1.2	45.5+	10.6+	1.8
13B	2700+0.5% Na ₂ B ₄ O ₇ · H ₂ O	4	3	263	166	6.1	0.7	0.9	11.0	1.8	0.9	35.0+	6.8+	1.1
14A	2700	8	6	235	148	4.0	0.6	1.1	6.9	1.4	0.7	17.9+	4.3+	1.1
14B	2700+1.0% Na ₂ B ₄ O ₇ · 10H ₂ O	9	6	262	140	5.5	0.5	0.4	8.7	0.9	0.1	23.4+	3.0+	0.3

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons for Groups 10A, 10B, 11A, 11B made with Group 9B and for Groups 13A, 13B, 14A and 14B with Group 12B.

than a developmental influence. This difference may be unimportant or it may be suggestive of the beginning of a trend that was more clearly evident in Group 11B. The average dental caries incidence of the offspring in Group 11A was not significantly different from the control rats in Group 9B. However, the post-developmental supplement of 1.0 per cent barium chloride in Group 11B caused highly significant reductions in the average number of carious areas and the average extent of caries scores and a significant reduction in the average number of carious molars. This 1 per cent level of barium chloride supplementation was the same that gave a less striking indication of a reduction in the maternal generation.

From these data it appears that these relatively high levels of barium chloride developmentally exerted no influence upon the developing teeth to alter the caries-susceptibility. However, a modest influence occurred post-developmentally to reduce the dental caries incidence. The fact that this trend was noted both in the maternal and filial generations tends to reinforce the importance of the observation even though the level of reduction was fairly low.

3. Sodium borate supplements

The third and fourth experiments were concerned with supplements of 0.5, 1.0 and 2.0 per cent sodium borate as listed in Tables 2 and 3. The rats in the post-developmental part of the maternal generation were sacrificed after 98 days in

Experiment 3 and 95 days in Experiment 4, while the offspring in the filial generation were sacrificed after 120 days on experiment.

The data for the post-developmental portion of Experiments 3 and 4 are presented in Table 2. The 0.5 and 1.0 per cent supplements of sodium borate had no influence on the rates of growth of male and female rats. However, the 2.0 per cent supplement caused very serious growth retardation; the condition of these rats was sufficiently precarious throughout that it was doubtful that they would survive the experimental period. Hence no attempt was made to breed animals at the 2 per cent level of sodium borate supplementation.

The 0.5 and 1.0 per cent sodium borate supplements caused modest reductions in the dental caries incidence which were in general statistically significant at the 5 per cent level. The 2.0 per cent supplement of sodium borate resulted in minor reductions in dental caries incidence that were not statistically significant. In view of the extremely poor physical condition of the latter rats, it would be difficult to attribute any change or lack of change in the dental caries incidence to the supplement. Furthermore, the lack of a significant reduction at the 2 per cent level should not negate the positive finding at the lower levels in view of the complicating physical condition.

The data for the developmental studies on sodium borate supplementation are given in Table 3. The 0.5 and 1.0 per cent sodium borate supplements did not influence the breeding ability of the rats detectably. There was a suggestion that the male experimental offspring were less thrifty and grew more slowly than the controls, but no evidence of impairment was noted among the females. While modest increases in dental caries incidence were observed in Groups 13A and 13B with the 0.5 per cent supplement of sodium borate during pregnancy and lactation, these increases were not of statistical significance. Furthermore, the 1.0 per cent supplement during pregnancy and lactation had no influence on the dental caries incidence. The post-developmental comparison between Groups 13A and 13B indicated a slight but not significant benefit of the 0.5 per cent sodium borate. The comparable comparison between control Group 14A and Group 14B with the 1.0 per cent sodium borate supplement did not indicate any post-developmental benefit for the experimental rats.

The data from the maternal and filial generations with respect to post-developmental influences of sodium borate supplements were not entirely consistent. If any post-developmental influence of 0.5 and 1.0 per cent levels of sodium borate supplementation occurred, it was at best a very mild one that may not be reproducible from generation to generation in the same population of rats. No convincing evidence of any trend toward an influence of sodium borate supplements during tooth development upon caries-susceptibility was observed.

4. *Lithium carbonate supplements*

The fifth experiment concerned supplements of lithium carbonate and was composed of Groups 17 and 18 which were fed diet 2700 and 2700+0.1 per cent lithium carbonate, respectively. Pilot studies had shown that 0.2 per cent and 0.5 per cent

lithium carbonate were too toxic to permit the rats to complete the experimental period. The rats in the maternal generation were sacrificed after 125 days on experiment and those in the filial generation after 95 days on experiment.

TABLE 4. THE POST-DEVELOPMENTAL INFLUENCE OF SUPPLEMENTS OF LITHIUM CARBONATE AND STRONTIUM CARBONATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
17	2700	12	12	211	124	5.9	0.6	—	9.7	1.2	—	27.9+	4.3+	—
18	2700+0.1% Li ₂ CO ₃	11	10	178	120	4.0	0.5	2.4	6.2	1.1	2.2	16.9+	3.2+	2.1
19	2700	4	4	209	120	7.9	0.9	—	13.3	2.0	—	35.6+	6.5+	—
20	2700+0.1% SrCO ₃	7	4	207	126	6.5	1.3	0.9	10.5	2.1	1.0	29.3+	6.2+	0.7
21	2700	13	10	238	132	6.7	0.6	—	11.8	1.4	—	34.3+	4.6+	—
22	2700+0.5% SrCO ₃	11	12	242	133	6.1	0.6	0.7	9.7	1.3	1.1	25.7+	3.9+	1.4
23	2700	10	9	235	120	5.4	0.6	—	9.8	1.4	—	30.6+	4.8+	—
24	2700+1.0% SrCO ₃	9	11	199	132	5.1	0.6	0.4	8.6	1.3	0.6	24.9+	4.7+	0.8
25	2700	3	3	179	113	4.0	1.0	—	6.5	1.5	—	23.2+	6.1+	—
26	2700+2.0% SrCO ₃	2	4	72	73	2.7	0.6	1.1	3.7	0.9	1.6	8.8+	2.4+	2.2

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons for Group 18 made with Group 17, for Group 20 with Group 19, for Group 22 with Group 21, for Group 24 with Group 23 and for Group 26 with Group 25.

The post-developmental data for this experiment are presented in Table 4. The rate of growth of the male rats receiving 0.1 per cent lithium carbonate was slowed appreciably, while the females were not affected. The dental caries incidence of the supplemented rats was reduced by amounts that were of statistical significance at the 5 per cent level.

The developmental data for the fifth experiment are presented in Table 5. Reproductive ability among the rats supplemented with 0.1 per cent lithium carbonate through the reproductive cycle was not adversely affected. The rate of growth of the offspring was not influenced among the rats in Group 18A whose parents received the 0.1 per cent lithium carbonate supplement but who received diet 2700 after weaning. However, their male littermates in Group 18B who continued to receive this supplement were penalized slightly. There was a slight suggestion that the females also were penalized. The incidence of dental caries was significantly increased among the rats in Group 18A which received diet 2700 after weaning but whose parents had received the 0.1 per cent lithium carbonate supplement. This increase compared to the value for the controls was significant at the 1 per cent level for the average number of carious molars and for the average extent of caries scores and was

significant at the 5 per cent level for the average number of carious areas. However, when the 0.1 per cent lithium carbonate was continued post-developmentally in Group 18B, the incidence of dental caries observed was almost identical to that of the controls with respect to the number of carious areas and the extent of caries scores and both values were much reduced from the values for the littermates in Group 18A. With respect to the number of carious molars, the situation was somewhat different with the numbers of carious molars for Groups 18A and 18B being similar and with the value for 18B being different from that for 17B by an amount of statistical significance at the 5 per cent level.

TABLE 5. THE DEVELOPMENTAL INFLUENCE OF SUPPLEMENTS OF LITHIUM CARBONATE AND STRONTIUM CARBONATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
17B	2700	14	14	281	157	6.0	0.4	—	10.9	1.1	—	34.6+	4.2+	—
18A	2700	15	14	288	158	7.8	0.3	3.6	15.1	1.0	2.8	53.8+	4.2+	3.2
18B	2700+0.1% Li ₂ CO ₃	15	16	264	130	7.5	0.5	2.3	11.3	1.2	0.3	39.5+	5.0+	0.8
21B	2700	16	15	277	169	6.4	0.4	—	11.1	1.0	—	34.3+	4.0+	—
22A	2700	10	15	291	173	6.6	0.3	0.4	12.9	0.9	1.3	48.4+	4.3+	2.4
22B	2700+0.5% SrCO ₃	11	13	289	164	7.0	0.4	1.0	13.8	1.1	1.9	49.3+	4.7+	2.4
23B	2700	19	27	268	174	5.3	0.3	—	8.9	0.7	—	28.6+	2.7+	—
24A	2700	9	11	275	159	8.9	0.5	6.2	17.8	1.1	6.8	65.2+	5.7+	5.8
24B	2700+1.0% SrCO ₃	7	9	280	166	8.8	0.5	6.0	16.9	1.5	4.8	62.8+	7.3+	4.3

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons made between Groups 18A, 18B and 17B, between Groups 22A, 22B and 21B and between 24A, 24B and 23B.

These data suggest that this 0.1 per cent supplement of lithium carbonate exerted a definite post-developmental influence to inhibit dental caries partially in both the maternal and filial generations. However, the same supplement during tooth development caused an increase in caries-susceptibility which could be largely overruled by continuation of the supplement post-developmentally. This reverse type of effect depending upon the time that the supplement was provided needs further investigation.

5. Strontium carbonate supplementation

Experiments 6–9 were designed to test the influence of supplements of 0.1, 0.5, 1.0 and 2.0 per cent strontium carbonate developmentally and post-developmentally as shown in Tables 4 and 5. The rats in the maternal generation of Experiments 6 and 9 were maintained for 98 days, while those in the filial generation were kept on the study for 120 days after weaning.

The data for the post-developmental part of the experiments are presented in Table 4. Post-developmentally, levels of 0.1 and 0.5 per cent strontium carbonate did not influence the rate of growth of the rats. The 1.0 per cent level resulted in a definite reduction in the growth rate of males but had no influence on the females. The 2.0 per cent level caused a major retardation in the rate of growth in both sexes.

The dental caries incidence among the experimental rats was slightly decreased by each of the four levels of supplementation. At the lower three levels, the reduction in any group was not significant at the 5 per cent level. At the 2 per cent level of strontium carbonate the decrease in dental caries incidence was only significant for the average extent of caries score at the 5 per cent level. While the majority of the differences was individually statistically insignificant, the common trend to a reduction in the supplemented groups is worthy of further study. However, it should be remembered that the penalty in rate of growth at the 2 per cent level of strontium carbonate was sufficiently great that caloric restriction may have occurred with its caries-reducing influence.

The data for the developmental influence of strontium carbonate are presented in Table 5. No animals were selected for breeding from Groups 19 and 20 since much higher levels of strontium carbonate could be tolerated readily. No animals were selected from Groups 25 and 26, since the 2.0 per cent level was so evidently toxic that reproduction was thought to be unlikely to occur.

The addition of 0.5 per cent or of 1.0 per cent strontium carbonate during pregnancy and lactation resulted in no interference in reproductive ability. In addition, the experimental offspring of both sexes grew and developed as well as those from control parents. The 0.5 per cent supplement of strontium carbonate during pregnancy and lactation resulted in modest increases in the dental caries incidence of the offspring in Groups 22A and 22B as compared to the controls in Group 21B. These increases were not statistically significant for the average number of carious molars and the average number of carious areas, but the increases for the average extent of caries score were significant in both Subgroups A and B at the 5 per cent level. The same trend was continued in the comparison of control Group 23B to Groups 24A and 24B where the parents had received 1.0 per cent strontium carbonate during pregnancy and lactation. However, in the latter comparison the increases were much more striking, with the critical ratios indicative of significance at the 1 per cent level. In the post-developmental comparisons that were possible between Subgroups 22A and 22B and between 24A and 24B, there was no evidence that supplements of strontium carbonate could counteract the increased susceptibility induced by the developmental administration of strontium carbonate. The latter observation was of interest in that it differed from that made in the maternal generation with respect to the post-developmental influence of strontium carbonate supplements.

6. *Vanadium pentoxide and vanadyl sulphate supplementation*

Experiments 10 and 11 were included to test the developmental and post-developmental influences of vanadium. In the four groups, 27-30, in Experiment 10, vanadium pentoxide was tested in the drinking water at levels of 18 and 36 p.p.m.

and in the diet at 18 p.p.m. These levels of vanadium pentoxide provided 5 and 10 p.p.m. of vanadium in the drinking water and 5 p.p.m. of vanadium in the diet. The rats in the maternal generation were maintained on experiment for 120 days while those in the filial generation were killed 140 days after weaning.

TABLE 6. THE DEVELOPMENTAL INFLUENCE OF SUPPLEMENTS OF VANADIUM PENTOXIDE AND VANADYL SULPHATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
27A	2700 (18 p.p.m. V_2O_5 in H_2O)	1	1	240	136	12.0	0.0	—	25.5	1.8	—	84.5+	5.9+	—
27B	2700	5	5	270	167	11.7	0.2	—	25.3	0.7	—	84.7+	6.8+	—
28A	2700	3	3	348	217	10.8	0.6	1.4	18.5	2.6	2.5	42.0+	7.1+	4.3
28B	2700 (18 p.p.m. V_2O_5 in H_2O)	1	1	309	175	9.5	0.4	—	17.0	0.9	—	33.5+	2.5+	—
29A	2700	5	3	349	189	12.0	0	1.5	23.4	1.0	1.6	60.6+	6.4+	2.6
29B	2700 (36 p.p.m. V_2O_5 in H_2O)	1	0	330	—	12	—	—	24	—	—	52+	—	—
30A	2700	12	11	303	186	10.6	0.5	2.8	20.5	1.1	2.4	61.7+	6.0+	2.7
30B	2700+18 p.p.m. V_2O_5	12	15	296	186	11.4	0.2	1.1	23.6	1.0	1.4	75.3+	5.0+	1.1
31A	2700 (16 p.p.m. $VOSO_4$ in H_2O)	2	1	279	167	12.0	0	—	26.3	0.8	—	110.0+	7.9+	—
32A	2700	8	7	297	168	12.0	0	—	26.7	0.4	—	111.0+	3.9+	—
32B	2700 (16 p.p.m. $VOSO_4$ in H_2O)	11	9	254	165	12.0	0	—	26.9	0.2	—	117.1+	2.7+	—
33B	2700+(32 p.p.m. $VOSO_4$ in H_2O)	1	0	175	—	12	—	—	24	—	—	59+	—	—
34A	2700	1	1	315	149	12.0	0	—	26.5	1.1	—	80.5+	11.1+	—
34B	2700+16 p.p.m. $VOSO_4$	2	0	275	—	12.0	0	—	24.5	0.4	—	75.5+	2.5+	—

* SEM—Standard error of mean.

† CR—Critical ratio. Comparison made between Groups 28A, 29A, 30A, 30B and 27B. No comparisons made in last half of experiment because of small numbers of rats in groups.

The data for the eight to ten rats in each group of the post-developmental halves of the experiments are not presented since they were mostly negative. Post-developmental supplements of vanadium provided as vanadium pentoxide or vanadyl sulphate at the above levels in drinking water and in the diet had no detectable influence upon the incidence of experimental dental caries in these trials. Body weights were not recorded in this maternal generation of these two experiments.

The data for the developmental portions of these experiments are presented in Table 6. Unexpectedly, the rate of reproduction was unpredictable and in general

inadequate in these experiments, even in the control groups. Reproduction was especially poor in Experiment 11, where inadequate offspring were available in most groups for a statistical comparison. In Experiment 10, the ten offspring of the control rats in Group 27 which were continued on diet 2700 had a rather high dental caries incidence for this strain under these experimental circumstances. The six rats in 28A whose parents had received 18 p.p.m. vanadium pentoxide in the drinking water throughout the reproductive cycle had less dental caries than the controls in Group 27B. This reduction was not significant for the number of carious molars, but was significant at the 5 per cent level for the number of carious areas and at the 1 per cent level for the extent of caries score. This type of response with no effect on the number of carious molars, a modest effect on the number of carious areas and an extensive effect on the extent of caries score has been encountered with the greatest rarity in our experimental procedures. The eight offspring in Group 29A whose mothers had received twice as high a level of vanadium pentoxide in the drinking water as those in Group 28A had a lower extent of caries score than that of the controls. This difference from the control values was statistically significant at the 5 per cent level, whereas no statistically significant difference was noted for the number of carious molars and the number of carious areas. In addition, it should be noted that the dental caries incidence among the rats in Group 29A was appreciably higher than for those in 28A, even though the mothers of rats in Group 29A received twice as much vanadium. The data for the dental caries incidence of the rats in Group 30A whose parents had received 5 p.p.m. of vanadium as vanadium pentoxide in the diet closely paralleled the data for Group 29A. Thus there were trends toward less experimental dental caries among the rats whose parents received vanadium during the reproductive cycle. However, it is unusual that the higher dosage level in Group 29A had less effect than the dosage level in Group 28A. This response to the graded doses suggests the presence of some other unrecognized variable. Another unexpected and unexplained observation concerned the higher rates of growth among the vanadium-supplemented rats.

As in the previous experiments, post-developmental comparisons can be made between 27A and 27B, between 28A and 28B, between 29A and 29B and between 30A and 30B. In all four comparisons, there is no suggestion of a post-developmental benefit of the supplementation with vanadium pentoxide.

In Experiment 11, only three offspring were obtained in the control group which had an unusually high incidence of dental caries for this population of rats. Among Groups 32A and 32B whose parents received 16 p.p.m. vanadyl sulphate throughout the reproductive cycle, good reproduction occurred and the offspring had high caries incidences comparable to those observed in the three control rats. For Groups 33B, 34A and 34B, a total of five offspring reached the normal time of termination and these had reduced incidences of dental caries. However, their numbers are so few that no comparisons can be made. It is interesting and a cause for speculation that the thirty-five rats in Groups 32A and 32B had such high dental caries incidences in the presence of vanadium supplementation in the parental generation.

The uncertainty of the results for the developmental influence of vanadium supplements coupled with the lack of sufficient offspring in these two experiments indicates the need for further investigation and suggests that some important uncontrolled variable may have been superimposed upon these experimental conditions. Certainly on the basis of these data, there is no evidence that post-developmental supplements of vanadium as vanadium pentoxide or as vanadyl sulphate in the diet or in the drinking water influenced the dental caries incidence in these populations.

7. *Ammonium molybdate and ammonium paramolybdate supplementation*

Experiments 12 and 13 were concerned with the developmental and post-developmental influences of supplements of 0.01 and 0.02 per cent ammonium molybdate, and 0.1 and 0.2 per cent ammonium molybdate, respectively. In Experiment 14, supplements of 0.01 per cent and 0.02 per cent ammonium paramolybdate were tested and in Experiments 15 and 16, supplements of 0.1 per cent and 0.2 per cent ammonium paramolybdate, respectively, were evaluated. The rats in the maternal generation were maintained on experiment for 90 days, while those in the filial generation were sacrificed after 110 days on experiment.

The data for the post-developmental influences of these supplements are presented in Table 7. The supplements of 0.01 per cent and 0.02 per cent ammonium molybdate for Groups 36 and 37 in Experiment 12 caused reductions in dental caries incidence post-developmentally that were statistically significant at the 1 per cent level for the number of carious molars and the number of carious areas and at the 5 per cent level for the extent of caries score. The rats in Experiment 13 were in general less caries-susceptible and more variable. The rats in Group 39 with the 0.1 per cent ammonium molybdate supplement had about a 50 per cent reduction in dental caries incidence as compared to the controls. However, because of the variability this reduction was not statistically significant. The reverse was true for the rats in Group 40 with a 0.2 per cent ammonium molybdate supplement where an increased dental caries incidence was observed. In the latter group, the level of molybdate administered was sufficient to produce definite toxic signs with respect to the rate of gain in body weight, especially in the males, whereas the lower levels had less effect upon the body weights. It is conceivable that the increased dental caries incidence in the rats in Group 40 may have been related to this toxicity. In Experiments 14-16, where ammonium paramolybdate was fed at different levels from 0.01 to 0.2 per cent in the diet to Groups 42, 43, 46 and 47, there was no evidence of any post-developmental influence on the dental caries incidence. Again the 0.2 per cent level was beginning to cause toxic manifestations with respect to gain in body weight, especially among the males, although the toxicity here was less than with the same level of ammonium molybdate.

The data on the developmental portions of Experiments 12 and 14 are presented in Table 8. In Groups 37A and 37B of Experiment 12 where a developmental supplement of 0.02 per cent ammonium molybdate was added to the diet during the reproductive cycle, the fifteen experimental offspring in Group 37A had almost an identical dental caries incidence to the twenty-eight control rats in Group 35B. In

this experiment the comparison between the rats in Groups 37A and 37B indicated a post-developmental benefit of the 0.02 per cent ammonium molybdate supplement that was statistically significant at the 5 per cent level and that was very similar to the level of reduction observed in the first generation of this experiment.

TABLE 7. THE POST-DEVELOPMENTAL INFLUENCE OF AMMONIUM MOLYBDATE AND AMMONIUM PARAMOLYBDATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
35	2700	7	7	174	85	10.1	0.8	—	19.2	2.2	—	53.4+	7.6+	—
36	2700 + 0.01% (NH ₄) ₂ MoO ₄	6	9	154	74	6.0	0.6	4.1	8.9	1.1	3.8	29.5+	3.7+	2.8
37	2700 + 0.02% (NH ₄) ₂ MoO ₄	8	7	131	91	5.6	0.8	4.5	8.8	1.5	3.5	31.4+	5.8+	2.3
38	2700	4	6	179	93	4.0	1.0	—	6.5	1.5	—	23.2+	6.1+	—
39	2700 + 0.1% (NH ₄) ₂ MoO ₄	4	4	187	93	2.0	1.1	1.3	3.4	2.1	1.2	12.2+	7.6+	1.1
40	2700 + 0.2% (NH ₄) ₂ MoO ₄	4	6	101	72	6.2	1.0	1.6	9.7	1.9	1.3	32.7+	7.8+	1.0
41	2700	15	16	213	126	5.0	0.5	—	8.1	1.2	—	24.5+	4.6+	—
42	2700 + 0.01% (NH ₄) ₂ MoO ₄ ·4H ₂ O	10	9	193	123	5.5	0.5	0.8	8.7	1.2	0.4	24.5+	3.6+	0.1
43	2700 + 0.02% (NH ₄) ₂ MoO ₄ ·4H ₂ O	15	14	202	130	4.6	0.4	0.6	7.6	1.1	0.6	22.9+	4.1+	0.3
44	2700	9	8	297	164	6.2	0.5	—	11.9	1.2	—	42.0+	5.4+	—
45	2700 + 0.1% (NH ₄) ₂ MoO ₄ ·4H ₂ O	11	5	281	162	6.7	0.6	0.6	11.5	1.4	0.2	38.0+	4.8+	0.6
46	2700	8	6	247	180	7.9	0.6	—	15.4	1.3	—	55.1+	5.9+	—
47	2700 + 0.2% (NH ₄) ₂ MoO ₄ ·4H ₂ O	7	7	199	162	7.9	0.8	0	14.7	1.5	0.4	56.1+	7.0+	0.1

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons are made between Groups 36, 37 and Group 35; between Groups 39, 40 and Group 38; between Groups 42, 43 and Group 41; between Group 45 and Group 44 and between Group 47 and Group 46.

In Experiment 14, supplements of 0.01 and 0.02 per cent ammonium paramolybdate were used during the reproductive cycle. The eleven experimental rats in Group 42A whose mothers had received the 0.01 per cent ammonium paramolybdate supplement during pregnancy and lactation had almost identical dental caries incidences to the eleven control rats in Group 41B. However, the thirteen experimental rats in Group 48B whose mothers had received twice as much molybdate through pregnancy and lactation experienced appreciably higher incidences of dental caries. These increases were significant at the 5 per cent level of significance for the number of carious lesions and at the 1 per cent level of significance for the extent of

caries score. Again in Experiment 14, the post-developmental comparisons between 41A and 41B, between 42A and 42B and between 43A and 43B did not indicate any influence of the ammonium paramolybdate supplements as was the case in the first half of this experiment.

TABLE 8. THE DEVELOPMENTAL INFLUENCE OF AMMONIUM MOLYBDATE AND AMMONIUM PARAMOLYBDATE ON THE INCIDENCE OF DENTAL CARIES IN WHITE RATS

Group	Diet	No. of rats		Avg. weight increase (g)		No. of carious molars			No. of carious areas			Extent of caries scores		
		♂	♀	♂	♀	Avg.	SEM*	CR†	Avg.	SEM*	CR†	Avg.	SEM*	CR†
35B	2700	15	13	267	140	7.2	0.5	—	13.3	1.1	—	43.4+	4.2+	—
37A	2700	6	9	261	152	6.6	0.8	0.6	11.8	1.7	0.7	41.2+	6.9+	0.3
37B	2700+0.02% (NH ₄) ₂ MoO ₄	8	8	253	149	4.9	0.8	2.4	7.9	1.5	2.9	26.8+	5.7+	2.3
41A	2700+0.02% (NH ₄) ₂ MoO ₄ ·4H ₂ O	4	6	249	163	4.3	1.0	0.6	6.9	1.8	0.7	21.5+	5.8+	0.5
41B	2700	4	7	281	148	5.1	0.8	—	8.6	1.8	—	25.3+	5.0+	—
42A	2700	4	7	266	158	5.7	0.6	0.6	8.7	1.5	0	25.0+	5.3+	0
42B	2700+0.01% (NH ₄) ₂ MoO ₄ ·4H ₂ O	6	8	272	140	6.0	0.5	1.0	11.4	1.6	1.2	36.7+	5.5+	1.5
43A	2700	7	6	274	172	6.9	0.5	1.9	14.9	1.4	2.8	48.9+	5.2+	3.3
43B	2700+0.02% (NH ₄) ₂ MoO ₄ ·4H ₂ O	6	5	272	163	6.8	0.5	1.8	11.9	1.3	1.5	35.4+	5.1+	1.4

* SEM—Standard error of mean.

† CR—Critical ratio. Comparisons are made between Groups 37A, 37B and Group 35B, between Groups 41A, 42A, 42B, 43A, 43B and Group 41B.

Weight increases among the rats in all the developmental trials with ammonium molybdate and ammonium paramolybdate did not indicate any toxic influence. Likewise, breeding proceeded at normal rates in all the supplemented and control groups.

The different response of the rats to the ammonium molybdate and ammonium paramolybdate supplements both during development and post-developmentally was remarkable. In general the supplements of ammonium molybdate post-developmentally were capable of producing modest reductions in the dental caries incidence of our strain of caries-susceptible rats. However, in the same circumstances, comparable supplements of ammonium paramolybdate were ineffective. Moreover, the supplementation of the diet with ammonium paramolybdate after the teeth have formed had no influence upon the incidence of experimental dental caries, whereas a supplement of 0.02 per cent ammonium paramolybdate during tooth development had a tendency to increase the caries-susceptibility of the offspring. Possibly the ionic state or the presence of impurities in these compounds may have influenced the results in these presently unexplained ways described above.

DISCUSSION

These developmental and post-developmental studies demonstrate the range of responses in dental caries incidence that can be elicited by dietary supplements of salts of various elements. Some of the compounds tested contained elements, barium, strontium and vanadium, that had been evaluated previously by other investigators in post-developmental studies conducted under other circumstances. Other salts contained elements that were of possible nutritional significance in the animal kingdom, such as molybdenum and boron.

In general, the various levels of sodium chloride, crude sea salt mixtures, barium chloride, vanadium pentoxide, vanadyl sulphate and ammonium paramolybdate had no post-developmental influence upon the incidence of experimental dental caries. However, under some circumstances barium chloride appeared to be capable of producing modest reductions in dental caries incidence. Sodium borate and strontium carbonate supplements post-developmentally caused modest reductions in dental caries incidence that were not consistently repeatable from one experimental situation to another. In contrast, lithium carbonate supplements caused consistent reductions that were significant at the 5 per cent level while ammonium molybdate supplements post-developmentally caused reductions that were statistically significant at the 1 per cent level.

Among these post-developmental tests, our results differ with respect to the influence of the strontium, vanadium and barium compounds from some other results that have been reported in the literature. RYGH (1949, 1950) reported that supplements of in the neighbourhood of 5-8 p.p.m. of vanadium pentoxide or vanadium trichloride and 0.012-0.014 per cent of strontium sulphate or strontium nitrate singly or together caused major reductions in the dental caries incidence of rats when added to a diet deficient in these elements. A 0.3 per cent barium chloride supplement alone or with zinc and thallium supplements was reported to cause major increases in the dental caries incidence. The descriptions of RYGH's experiments are rather vague and at times contradictory with respect to the levels of supplements, the specific salts used, and the degree of reduction in the dental caries incidence. The contradictions particularly appear between the two papers despite the indication from rat numbers that the same rats were represented in some groups of the experiments described in the two papers. In addition, no statistical evaluation of the differences was included. He also reported that strontium and vanadium supplements enhanced calcification while barium, zinc and thallium supplements decreased calcification. We observed no evidence to indicate that the strontium, vanadium and barium supplements had altered the rate or degree of calcification in our experimental populations. GEYER (1953) also reported that the addition of 0.04 or 0.08 mg of vanadium pentoxide to the diet of hamsters or the subcutaneous injection of 0.07 mg of vanadium pentoxide once weekly post-developmentally caused striking reductions in the dental caries incidence. In our experiments, neither the supplementation of the diet or the drinking water with vanadium pentoxide or vanadyl sulphate post-developmentally had any influence on the dental caries incidence of

rats. In this regard our results were consistent with the negative results of HEIN and WISOTZKY (1955) who provided 10 p.p.m. of vanadium as vanadium pentoxide in the drinking water of hamsters and MUHLER (1957) who provided either 10 or 20 p.p.m. of vanadium as vanadium pentoxide in the drinking water of rats.

The reductions observed in our experiments with post-developmental supplements of strontium carbonate were minor and rather inconsistent compared to those described by RYGH. Strontium chloride at a level of 50 p.p.m. of strontium in the drinking water of hamsters was tested by JOHANSEN and HEIN (1953) and found to be ineffective in altering the dental caries incidence post-developmentally. Likewise our results with barium chloride were fairly inconsistent and suggested a modest ability to reduce the dental caries incidence in contrast to RYGH's report of a striking increase in dental caries incidence. No other studies on barium salts are known to have been published.

The experiments reported by RYGH differed from all the others in that he had diligently sought to reduce the trace element content of the basal diet drastically. His claim that the experimental diet was "absolutely free from all traces of inorganic nutritive trace elements" seems extravagant in the light of current evidence of the great difficulty of obtaining complete purity of dietary ingredients. Nonetheless, his diligent care in the preparation of his diet undoubtedly resulted in a different dietary baseline than that in any of the other studies. This difference in dietary procedure could explain the difference in the positive results with vanadium supplementation observed by RYGH versus the negative results reported by HEIN and WISOTZKY (1955), by MUHLER (1957) and in our laboratories. However, this explanation would not hold for GEYER's results, who with an ordinary diet obtained positive results with vanadium supplements.

During tooth development, tests strictly comparable to our experiments are not known to have been conducted with respect to experimental dental caries. The various levels of barium chloride, sodium borate and ammonium molybdate tested during tooth development had no influence upon the dental caries susceptibility of the offspring. Developmental supplements of vanadium pentoxide and vanadyl sulphate were inconclusive by reason of the poor reproduction encountered in these experiments. However, on the basis of the small numbers of offspring, there was little evidence that these vanadium supplements had influenced the dental caries susceptibility. Further studies need to be conducted with these developmental supplements of vanadium compounds.

However, developmental supplements of sodium chloride, a local sea salt preparation, Indian sea salt, Sambhar Lake salt, lithium carbonate, strontium carbonate and ammonium paramolybdate caused significant increases in the dental caries-susceptibility of the offspring at levels varying from 5 per cent to 1 per cent. The varied nature of these supplements and the fact that they uniformly caused increases rather than decreases in susceptibility to dental caries suggest that there is probably a desirable metabolic environment for the development of teeth and a desirable elemental composition for the teeth, neither of which can be deviated from materially without adversely influencing the ability of the teeth to withstand a highly cariogenic

oral environment. It is worthy of note that these metabolic circumstances were brought about under sufficiently mild circumstances that normal reproduction occurred as well as fairly normal growth and development among the offspring. The mechanism whereby these increases in dental caries-susceptibility occurred as a result of various supplements cannot be delineated from these gross experiments. Additional more refined experiments will be necessary to provide more specific information.

The influence of the molybdenum salts in these trials is worthy of special query. Ammonium molybdate produced modest reductions in the post-developmental trials but was ineffective in the developmental evaluation; in distinct contrast, ammonium paramolybdate was ineffective in the post-developmental trials but was effective in increasing the dental caries-susceptibility in the developmental evaluation. These different results suggest that the valence of the elements in the compounds and the solubility of the compounds may be of primary importance. In addition, the possibility of varying impurities between two salts needs to be evaluated in future trials.

ADLER (1957) has reported very striking reductions in the dental caries incidence of rats when 0.1 p.p.m. of ammonium molybdate was provided in their drinking water, post-developmentally and during development. An experimental design somewhat similar to the one employed in our studies was used except for his use of a coarse corn diet instead of purified diet 2700, and of a relatively more caries-resistant strain of rats. In the developmental and the post-developmental trials, the males had greater caries reductions, 91.0 and 87.5 per cent, respectively, as compared to the females with reductions of 75.4 and 56.6 per cent, respectively. The differences between our studies and ADLER's cannot be explained, especially in view of the very small amount of molybdenum provided to his experimental rats.

KRUGER (1959) has reported the results of a series of six experiments in which compounds of various elements were injected intraperitoneally into rats daily between the fifth and seventeenth days of age to determine whether the caries-susceptibility of the developing first and second molars could be influenced. In the first three experiments, simple direct designs were used. The daily injection of 0.15 mg of manganese as manganese sulphate was ineffective. The daily injection of a mineral salt solution providing 0.005 mg boron, 0.0005 mg copper, 0.002 mg of molybdenum, 0.054 mg fluoride and 0.05 mg of manganese was reported to cause a reduction in caries incidence expressed as gross lesions before grinding that was significant at the 5 per cent level. However, when a detailed examination of the teeth was made, the number of lesions and the extent of lesions did not differ significantly between the control and supplemented groups. In a similar experiment, the four salts of boron, copper, molybdenum and manganese were injected alone at the above levels, the same amount of fluoride was injected in another group, and all five salts were injected into a third group. The group injected with the four salts did not differ from the controls. However, both of the other groups had significantly lower levels of caries than the controls at the 1 per cent level. This result is in striking contrast to the much less definitive finding in the previous experiment. It is interesting that the group with the fluoride supplement only had benefited appreciably more than

those given all five salts. Up to this point in KRUGER's studies, the experimental design was sound for the desired purpose of the trials and ample rats were present in each group. No convincing evidence of a benefit was reported for any other element or grouping of elements than fluoride.

The remaining three experiments had complicated, sophisticated designs in which interpretation had to be based solely on statistical data. Each treatment group contained one, two or three rats. As many variables as different compounds, different combinations of compounds and different dosage levels were evaluated simultaneously. Boron provided as boric acid, molybdenum as ammonium molybdate and vanadium as vanadium chloride were reported to have caused reductions in the dental caries-susceptibility. In actuality all three of these cases rest on slim evidence. For example, with boron, no significant difference was reported in one experiment where the highest level was 19.5 μg per day, while a significant difference at the 0.1 per cent level was reported from another experiment where 25 μg per day was injected. The case for vanadium rested on the caries incidence for two rats that received only the vanadium supplement, although thirty of the other sixty-four rats in the experiments received the vanadium supplement along with one, two, three or four other supplements. The source of vanadium was described only as vanadium chloride with no specification as to whether it was the mono-, di-, tri- or tetra-chloride. The case for molybdenum rested on two rats which had received only the molybdenum supplement and two rats which received the molybdenum supplement along with another supplement. From KRUGER's studies fluorine is the only element that can be reasonably confidently accepted as having produced a change in dental caries-susceptibility. Unfortunately, the lack of uniformity in caries-susceptibility among available strains of rats seems to preclude the use of as highly sophisticated experimental designs until much more predictable inbred strains have been developed.

Human studies concerning the majority of the elements evaluated in this series of experiments have yet to be made. However, from epidemiological evidence it has been suggested that vanadium and molybdenum may be related to the problem of dental caries in man. TANK and STORVICH (1960) reported a survey conducted in Oregon of the influence of naturally occurring selenium and vanadium on dental caries in areas with varying levels of fluoride in the drinking water supplies. They concluded that there appeared to be a trend toward decreasing caries activity with increasing vanadium concentrations in the water and that there was a significant decrease in dental caries rates for the permanent teeth with increasing vanadium intake. The numbers of children in the vanadium part of the study were relatively small. The study was further complicated by the necessity to consider simultaneous variations in selenium intake with the tendency to an increased caries incidence and variations in fluoride and vanadium with the trend toward decreased caries incidence. A low level of significance was observed for the differences in the permanent teeth when all groups with increased vanadium intake were combined. The survey concerning molybdenum was conducted in New Zealand by LUDWIG, HEALY and LOSEE (1960) in the nearby communities of Hastings and Napier where the children 5-8 years old in Napier had a considerably lower caries experience than those in Hastings.

Although these communities were similar in regard to such things as climatic environment, socio-economic conditions, racial composition and milk and water supplies, it was postulated that the children of Napier consumed more molybdenum from dietary sources than those of Hastings due to the growth of the vegetables in the Napier area on soils which have a recent marine history. ADLER and STRAUB (1953) and NAGY and POLYIK (1955) have also reported that there is an association between high molybdenum levels in the soils and water supplies and low caries prevalence in man.

Acknowledgements—This investigation was supported to a large extent by a PHS research grant D-204 from the National Institute of Dental Research, Public Health Service.

We are indebted to Dr. LYON P. STREAN, Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania, for generous supplies of the vitamin B complex provided for use in these experiments.

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SHORT COMMUNICATION

FURTHER OBSERVATIONS ON THE SUDAN BLACK STAIN FOR CALCIFICATION

J. T. IRVING and R. E. WUTHIER

Forsyth Dental Infirmary and Harvard School of Dental Medicine,
Massachusetts, U.S.A.

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In a previous communication (IRVING, 1959) a method was described for staining the sites where calcification was being initiated. In brief the method consisted, after formalin fixation, of extraction with hot pyridine, decalcification, embedding in gelatin and cutting the sections on a freezing microtome, in that order. The sections were stained with Sudan black B and mounted in glycerine jelly. The matrix surrounding hypertrophic cells in the epiphyseal cartilage, the junction of the preosseous matrix with calcified bone (the calcification line), and the junction of predentine and dentine stained intensely with Sudan black, the rest of the hard tissues being unstained.

At the time the method was first developed, it was considered that some form of mucopolysaccharide was involved, since the areas showing γ -metachromasia with toluidine blue and staining with Sudan black did to some extent coincide. WISLOCKI and SOGNAES (1950) had shown that Sudan black would stain the granules of mast cells. However, it was noted at the time that toluidine blue consistently stained a larger area than Sudan black around the hypertrophic cells of the epiphyseal cartilage; also that it stained cartilage remnants in the trabeculae of the primary spongiosa, and decalcified bone and dentine uniformly, but none of these sites were sudanophil. In addition, it was possible under certain conditions to remove the metachromasia around the hypertrophic cells with hyaluronidase, while the Sudan black staining was unchanged. Finally it was more recently found (IRVING, 1960) that after papain injections the metachromasia of the epiphyseal cartilage disappeared completely, but the Sudan black staining was unaffected. It was then concluded that the sudanophil substance was certainly not chondroitin sulphate, and probably not a mucopolysaccharide.

Some earlier observations made it seem possible that a lipide substance was involved. Thus if the tissues, after pyridine extraction and decalcification, were embedded in paraffin instead of gelatin, the sudanophilia was lost. A certain variability in the results after pyridine extraction led to the consideration that pyridine extraction, if carried out too long, would remove the sudanophil material completely.

Sections from a rat tibia, which had been extracted with pyridine and decalcified, were stained with Sudan black, and exhibited the usual picture. They were then

extracted with pyridine for 6 hr, washed and restained. The sudanophil material of the epiphyseal cartilage had been completely removed, but that of the calcification lines still stained. Similar treatment with absolute alcohol, acetone, benzene, chloroform or petroleum ether reduced the staining of the epiphyseal cartilage, but had no effect on that of the calcification lines.

Two tibias were taken from a young rat, and were both extracted with pyridine and decalcified. One was stained in the usual way, but the other was replaced in hot pyridine (60°) for 3 days after decalcification. It was then embedded and stained. The first tibia stained as usual with Sudan black, but the second showed no staining at all of the epiphyseal cartilage or the calcification lines.

It was therefore concluded that the sudanophil substance is lipidic in nature, and that it is soluble in pyridine, but the material in the epiphyseal cartilage is more soluble than that in calcifying bone. Also that the material is a great deal less soluble in the other lipidic solvents which were tested.

As earlier reported, pyridine treatment must precede decalcification, otherwise the sudanophil material is removed with the inorganic constituents. Pyridine thus apparently removes first a substance which is not sudanophil but which attaches the sudanophil material to the inorganic phase, and in so doing unmasks this second substance so that it will stain with Sudan black.

FELS (1961) has concluded that a protein-phospholipid complex is responsible for calcium binding by the aorta, and the present results suggest similarly that a lipidic substance is associated with calcification in bone.

Acknowledgement—This project was supported by a Research Grant, D-876, from the National Institute of Dental Research.

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- I. L. DOGON, A. C. KERR and B. H. AMDUR: Characterization of an antibacterial factor in human parotid secretions active against *Lactobacillus casei*
(Forsyth Dental Infirmary, 140 The Fenway, Boston 15, Mass.)
- J. C. THONARD and H. W. SCHERP: Histochemical demonstration of acid mucopolysaccharides in human gingival epithelial intercellular spaces
(Department of Bacteriology, School of Dentistry, University of Pittsburgh, Pittsburgh 13, Pa.)
- J.-E. GLAS: Studies on the ultrastructure of dental enamel—II. Orientation of the apatite crystallites as deduced from X-ray diffraction
(Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden)
- P. J. WOOD and B. S. KRAUS: Prenatal development of the human palate: some histological observations
(Department of Orthodontics, School of Dentistry, University of Washington, Seattle, Wash.)
- D. C. A. PICTON: Tilting movements of teeth during biting
(University College Hospital, Dental School, London W.C.1)
- C. DAWES and G. N. JENKINS: Some inorganic constituents of dental plaque and their relationship to early calculus formation and caries
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- E. JOHANSEN and H. F. PARKS: Electron-microscopic observations on sound human dentine
(Department of Dentistry and Dental Research, University of Rochester, Rochester, N.Y.)
- A. R. TEN CATE: The distribution of alkaline phosphatase in the human tooth germ
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- F. W. KRAUS and S. SIRISINHA: Gamma globulin in saliva
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- G. R. SEWARD: The radiographical anatomy of the human lateral nasal wall in occlusal radiographs
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- P. H. KEYES and R. J. FITZGERALD: Dental caries in the Syrian hamster—XI. Studies on the fate of "labelled" cariogenic streptococci
(N.I.D.R., N.I.H., P.H.S., U.S. Dept. of Health, Education and Welfare, Bethesda 14, Md.)
- D. ADAMS: The blood supply to the enamel organ of the rodent incisor
(Anatomy Department, The University, Edinburgh)

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1. ANATOMY

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